Application of Statistics to Medicine, particularly the Study of Cell Proliferation

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1988

Abstract

The 120 articles comprising this submission were published between 1971 and 1988. Principal journals are *Cell and Tissue Kinetics*, *Virchows Archiv*, and the *British Journal of Cancer*. As a medical statistician, the candidate performed the data analysis, frequently participated in the design of the studies, and shares the responsibility for the interpretation of the results.

Many types of studies are represented, including analysis of patient records, epidemiological or hospital surveys, case-control studies, clinical trials, and validation of laboratory measurements, but more than half (66) are concerned with the study of cell proliferation, mainly in the rodent and human intestine.

Many types of analysis were undertaken, some no more than graphical presentation or the appropriate use of straightforward methods such as the t-test, simple linear regression and the analysis of two-dimensional contingency tables. Sometimes more sophisticated methods, such as discriminant analysis and unbalanced analyses of variance and covariance, were employed, and there is some curve fitting and substantial use of simulation, especially in the cell proliferation area.

Those 23 articles where the candidate is first or sole author tend to be either theoretical contributions to the study of cell proliferation, or didactic articles designed to help medical research workers understand medical statistics.

Acknowledgements

It is a pleasure to acknowledge that not all of the work reported in the publications which follow is my own. A medical statistician must rely heavily on his colleagues who gather the data he analyses; they count the cells, they see the patients, but he will be the first to know what inferences can be drawn from the data they collect. At least, he will if they have patiently explained the biological or medical problem to him, and I am grateful to my collaborators for doing this. I am also grateful to my present colleagues for their encouragement to make this submission.

No-one could have asked for more assistance than I have received from my pathologist friends: Nick Wright and Adrian Morley introduced me to the subject of cell proliferation, and Jim Sunter and Alex Watson (with many co-workers) have continued to keep me supplied with interesting problems of experimental design and analysis, and have listened to my views on more than the statistical aspects of the subject.

Special thanks must also go to two people who enabled me to deal not only with problems in experimental medicine but also with the epidemiological questions which often form the bulk of other medical statisticians' experience: Michael Tunbridge provided my first large data set, and Andrew Rugg-Gunn made sure that I would include dentistry as part of my territory. But I am grateful to all my co-authors, for they all took care to explain to me what they were investigating, in such a way that I have at least believed I understood. These articles represent more than the day-to-day consultancy problems which a medical statistician meets: the t-tests and regression analyses he performs as a matter of course after what is sometimes an all-too-cursory glance at the data he has been brought.

To all those who have worked with me in the Department of Medical Statistics at Newcastle University I extend my warmest thanks, in particular to David Newell who, as my head of department for 17 years, was a constant support.

Introduction

The list of publications forming the basis of this submission appears on pages 8-21, and the papers are categorised under some quite disparate and sometimes rather arbitrary headings, such as 'Cell Kinetic Theory' and 'Diet and Dentistry'. It may be worth taking a little time to explain this diversity by considering the nature of medical statistics and its relationship to the sciences it seeks to assist. This topic is discussed in some detail by Healy (1979) who points out that "the medical statistician will typically be involved in collaborative work" and that he must "know all about statistics – multivariate analysis, experimental design, stochastic processes, survey sampling, you name it – and a good deal of not only medicine but a lot of other areas in which statistical methods are applied."

I have found this requirement stimulating, but I believe that a medical statistician also needs to have a particular interest – for his own satisfaction as well as his career development. For some it may be an aspect of statistics, such as clinical trial design or analysis of survival data; for others it may be a medical problem, such as the effects of smoking on health or how a mother's blood constituents change during pregnancy. The first option keeps the medical statistician in the statistical fold, though it may make him susceptible to the ailment which afflicts many mathematical statisticians: that of indulging in theory less relevant to the needs of experimenters than it might be. The second, less frequently followed option produces a hybrid creature which is neither statistician nor laboratory worker nor clinician. He may be the international expert in the use of the programming language APL to simulate and hence analyse the production of lactic acid in pregnancy, but such a distinction is recognised only by a few of his obstetric colleagues, by fewer still of his statistical colleagues, and by hardly any of his other medical colleagues for whom he estimates the required sizes of clinical trials, performs analyses, and gives courses in the elements of medical statistics.

Nevertheless, I believe that the most challenging route for any applied statistician to take is to ally himself to a particular investigation and provide his experimental colleagues with all the necessary tools for designing, analysing and interpreting their experiments. Thus more than half of the publications which follow are on one topic: that of cell proliferation, particularly in the intestine. I have enjoyed the mathematical, statistical and computing problems which have arisen over the years, as I have enjoyed learning about the process of cell proliferation itself to the extent of editing (with colleagues, but as first author) a book on the subject. The reviews of this book appear on pages 6 & 7.

My interest in cell proliferation began when a model was required for the response to testosterone of the seminal vesicle of the castrate mouse; this definitive model was described and used in a series of papers [57-63]. The pattern of authorship is fairly typical: the data collector (rightly) is normally first author, and only when the model itself is being described does the statistician supplant him. Most of the cell proliferation studies have been of the gut [9,12-56]; one deals with adrenal cortex [64], one with stomach [66] and two [10,65], in dealing with stratified squamous epithelium (skin and oesophagus), propose a theory for cell proliferation and migration in such tissue. Some papers [5-8,11] deal with the theory of particular experimental techniques rather than with particular tissues, and four [1-4] are didactic: because of the influence he can wield the medical statistician possibly has a greater responsibility than other scientists to explain his attitudes and methods to his colleagues in other disciplines. It would have been possible to write more first-author papers in the cell proliferation area, but there are already too many theories looking for data; I have always tried to be driven by observations, not speculations.

The data leading to the other papers were, as indicated earlier, varied. Nineteen papers [71-75,102-114,117] came from two large epidemiological surveys, and the only other substantial group is a series of six, again didactic, on the analysis of data in nephrology [83-88], written by request. In total, I am first or sole author of 23 of the 120 papers.

All published papers are compromises: between the co-authors who will all see different aspects of the research from different angles, and between the authors as a group and the referees and editors who wish to protect not only the academic reputation of their journal, but also its particular style. Medical journals have paid more than lip service to statistics for many years now: so much so that it sometimes seems that one must have 'p-values' if a paper is to be suitable for publication. If only these were required rather less often and space to print graphs were found more often! In the publication list which follows those papers which appeared in a refereed journal are marked with an asterisk. A 10% sample of the articles, covering the whole period and reasonably representative in terms of authorship, journals, subject matter, method of analysis, and scientific approach can be obtained from numbers 3, 22, 44, 47, 52, 61, 65, 74, 88, 95, 114 & 115.

Reference

Healy, M.J.R. Does medical statistics exist? Bias 6 (1979) 137-182.

Book Published

Appleton, D.R., Sunter, J.P. & Watson, A.J. (eds)
Cell Proliferation in the Gastrointestinal Tract.
Pitman Medical, Tunbridge Wells, 1980.

Reviews

Cell & Tissue Kinetics 15 (1982) pp 575-576.

This book represents a very clear and comprehensive review of the cell kinetic aspects of the different sections of the intestine. It results from the papers presented at a conference in Newcastle in September 1979. The editors have succeeded in providing a surprising uniformity in the method of presentation by imposing a standardized nomenclature and by redrawing many of the Figures in a common format. They have also compiled all the references into a common bibliography of 838 references at the back of the book. These features make it much more useful than many conference proceedings and will be appreciated by most readers, although one can imagine some resistance may have been encountered from the contributors.

The small intestine was the first tissue to be investigated by cell kinetic techniques when tritiated thymidine and autoradiography became available two decades ago. It was shown to have a very rapid turnover of proliferating cells in the crypt and an orderly progression of cells through the stages of differentiation until their death at the tip of the villus. These presentations show how variable the proliferation rates can be at different positions within the crypt/villus population, in sickness and in health, and in response to treatment. Furthermore, the proliferation rates and the means by which a steady state balance between cell production and cell loss [is maintained] *are shown to be quite different in the different regions of the G.I. tract, both longitudinally as one descends from the stomach, and circumferentially within one region.*

The orderly progression of cells from the base of the crypt to the tip of the villus is shown to be a complex piece of choreography since some differentiated cells are moving in the opposite direction and the influence of underlying non-epithelial components is likely to be important.

The response to cytotoxic drugs, surgery, irradiation, variations in diet and even the time of the day are presented in considerable detail. The relative importance of hormones and other systemic factors compared with luminal factors is demonstrated. Changes occurring in pre-malignant and malignant states induced experimentally in rodents and occurring naturally in man also provide the subject matter for two of the six separate sections.

For students and experienced research workers alike this book provides a fascinating insight into the complexity of homeostatic control mechanisms within a single organ. The six review papers, which lead into the more detailed research presentations, are a valuable source of teaching material. The very large bibliography make it an excellent reference book for both students and specialists.

Since it seems likely that books of this kind may ultimately appear for each tissue and organ system in the body it is to be hoped that they will all be as comprehensive and interesting as this.

J. Denecamp

Journal of Anatomy 134 (1982) p134

This is a fascinating book and, for the specialist, a necessary addition to the bookshelf. It is the result of a symposium held in Newcastle in 1979 and is very much up to date. Unlike some published symposia the editors have gone a long way to produce a tidy, coherent volume rather than a transcript of the proceedings. Not only are the contributions well orchestrated but there is an above-average uniformity of style and, further, the editors have imposed a common terminology with great success. There is no discussion and all the references come at the end of the text; the bibliography alone would make the book a valuable acquisition. A minor blemish is the absence of summaries to some of the chapters. None the less this is a big step forward compared with many similar symposia that come to print.

A wide representation of workers in the field has been assembled, some presenting 'commissioned' papers reviewing critical areas while others contribute shorter communications. There are some excellent 'keynote' chapters, particularly on normal cell proliferation, by N.A. Wright, and on stem cells, by C.S. Potten. The volume has six sections, beginning with normal proliferation, followed by growth control, stem cells and the response to cytotoxic agents,experimental carcinogenesis and, finally, human studies in health and disease. Inevitably the firm and comprehensive handling of the opening chapters cannot be maintained and some of the shorter contributions are more tentative and preliminary. However, the book shows the existing way in which the field is developing and, for the clinician, indicates the immense potential of knowledge in this field.

In summary, this is a book that should be studied by both basic and clinical workers and may well stimulate new ventures.

B.Creamer

PUBLISHED ARTICLES

CELL PROLIFERATION

Cell Kinetic Theory

1. **Appleton, D.R.**
* Aspects of statistics in studies of cell proliferation. I Multistage sampling.
Cell & Tissue Kinetics **17** (1984) 545-548.

2. **Appleton, D.R.**
* Aspects of statistics in studies of cell proliferation. II Standard errors of parameter estimates.
Cell & Tissue Kinetics **17** (1984) 657-659.

3. **Appleton, D.R.**
* Aspects of statistics in studies of cell proliferation. III Alternative models give different results.
Cell & Tissue Kinetics **18** (1985) 111-113.

4. **Appleton, D.R.**
* Aspects of statistics in studies of cell proliferation. IV Simulation.
Cell & Tissue Kinetics **18** (1985) 133-135.

5. Gratton, R.J., **Appleton, D.R.**, & Alwiswasy, M.K.
The measurement of tumour growth rates.
In 'Biomathematics and Cell Kinetics', ed. Valleron, A-J. & Macdonald, P.D.M., Elsevier/North Holland (1978) 211-223.

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The robustness of methods of analysing stathmokinetic experiments.
In 'Mathematical Models in Cell Kinetics', ed. Hartman, N.R., European Press (1975) 49-51.

7. Wright, N.A. & **Appleton, D.R.**
* The metaphase arrest technique: a critical review.
Cell & Tissue Kinetics **13** (1980) 643-663.

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* Estimating the proportion of proliferating cells in a population.
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* The highest labelled cell in the intestinal crypt column.
Cell & Tissue Kinetics **21** (1988) in press.

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Journal of Theoretical Biology **65** (1977) 769-779.

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The fraction labelled mitoses method: general considerations.
In 'Psoriasis: Cell Proliferation', ed. Wright, N.A. & Camplejohn, R.S., Churchill Livingston (1983) 24-33.

The Normal Rat & Mouse Small Intestine

12. Al-Dewachi, H.S., Wright, N.A., **Appleton, D.R.**, & Watson, A.J.
* The cell cycle time in the rat jejunal mucosa.
Cell & Tissue Kinetics **7** (1974) 587-594.

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* Cell population kinetics in the rat jejunal crypt.
Cell & Tissue Kinetics **8** (1975) 361-368.

14. Wright, N.A., Morley, A.R., **Appleton, D.R.**, & Watson, A.J.
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Cell & Tissue Kinetics **5** (1972) 351-364.

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* Studies on the mechanism of diurnal variation of proliferative indices in the small bowel mucosa of the rat.
Cell & Tissue Kinetics **9** (1976) 459-467.

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* Variation in the cell cycle time in the crypts of Lieberkuhn of the mouse.
Virchows Archiv B **31** (1979) 37-44.

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Virchows Archiv B **18** (1975) 225-242.

The Normal Rat & Mouse Colon

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* Cell proliferation at different sites along the length of the rat colon.
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Journal of Anatomy **149** (1986) 177-188.

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Virchows Archiv B **29** (1979) 351-361.

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Journal of Anatomy **129** (1979) 833-842.

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Cell proliferation in the mouse large bowel, with details of the analysis of the experimental data.
In 'Cell Proliferation in the Gastrointestinal Tract', ed. **Appleton, D.R.**, Sunter, J.P., & Watson, A.J., Pitman Medical (1980) 40-53.

Adaptation of the Rat & Mouse Intestine to Abnormal Conditions

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Journal of Anatomy **119** (1975) 105-121.

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Cell & Tissue Kinetics **10** (1977) 203-213.

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Virchows Archiv B **28** (1978) 339-350.

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Cell & Tissue Kinetics **17** (1984) 445-452.

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Stathmokinetic studies on intestinal crypts following 70% resection of the small bowel or transplantation of 10-20 mm of small bowel to the exterior abdominal wall.
In 'Mechanisms of Intestinal Adaptation', ed. Robinson, J.W.L., Dowling, R.H., & Riecken, E-O., MTP Press (1982) 337-351.

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Cell & Tisue Kinetics **19** (1986) 491-502.

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Journal of Pathology **146** (1985) 39-49.

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* A trophic effect of epidermal growth factor (EGF) on rat colonic mucosa in organ culture.
Cell & Tissue Kinetics **20** (1987) 43-56.

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Virchows Archiv B **34** (1980) 299-309.

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Autoradiographic investigations of cell proliferation in the small and large bowel of the mouse, and the jejunal response to some abnormal conditions.
Acta Histochemica **27** (Suppl., 1983) 185-194.

Carcinogenesis and Tumours in the Rat & Mouse Intestine

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* Pathological features of the colonic tumours induced in rats by the administration of 1,2-dimethylhydrazine.
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* Kinetics of changes in the crypts of the jejunal mucosa of dimethylhydrazine-treated rats.
British Journal of Cancer **37** (1978) 662-672.

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Journal of Pathology **156** (1988) in press.

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Gut **14** (1973) 603-606.

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Journal of Clinical Pathology **32** (1979) 462-470.

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Cell & Tissue Kinetics **15** (1982) 555-564.

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Cell & Tissue Kinetics **18** (1985) 273-277.

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The Mouse Prostate

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In 'The Cell Cycle in Development and Differentiation', ed. Balls, M. & Billet, F.S., CUP (1973) 337-340.

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Journal of Endocrinology **65** (1975) 353-361.

Other Tissues

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TOPICS OTHER THAN CELL PROLIFERATION

Endocrinology

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Lancet **2** (1971) 279-283.

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Clinical Endocrinology **2** (1973) 369-376.

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Cell Tissue Kinet. (1984) **17**, 545–548.

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Aspects of statistics in studies of cell proliferation

I. Multistage sampling

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(*Received* 12 *September* 1983; *revision accepted* 28 *December* 1983)

Abstract. In cell kinetic experiments, as in many other branches of biological science, we often sample at various stages of an experiment: we may for example take a sample of animals, then from each study a sample of sites, and from each site take replicate observations. This sampling process can be optimized to give maximum precision to an estimated quantity, but care must be taken in analysing data so gathered because the analysis depends on the precise sampling strategy.

The purpose of this series of short didactic articles is to illustrate, by example, the part statistics can play in the design, analysis and interpretation of cell kinetic experiments, and how failure to apply the correct statistical technique may lead to error. This particular article is concerned with the analysis of experiments in which sampling occurs at several stages. The first example shows how the design of an experiment can be optimized in that it produces a minimum standard error for the quantity to be estimated, for a given amount of counting. The second example shows a very common error in such designs (Gore, Jones & Rytter, 1977), that of ignoring one source of variability; this may lead to the application of quite erroneous statistical tests.

Example 1

Suppose an experiment involves studying the *in vitro* response of a tissue to some agent administered *in vivo*, for example how intestine, rendered hyperplastic by administration of dimethylhydrasine (DMH) to rats, proliferates in culture. We wish to know how many animals to use, how many blocks to take from each animal, and how many cultures to set up from each block. Of course the procedure is necessarily much complicated since we do not know that all animals will develop tumours, nor that each culture will be successfully maintained for the required time, but we can nevertheless consider how to obtain maximum precision from one experiment. Suppose that we are measuring some index of proliferation x, and that we choose n_A animals, n_B blocks from each animal, and n_C cultures from each block; suppose also that the variance between cultures within the same block is σ_C^2, that the variance between the mean values of blocks within the same animal is σ_B^2 and that the variance between mean values of different animals is σ_A^2. The standard error of the overall mean is:

$$\{\sigma_A^2/n_A + \sigma_B^2/n_A n_B + \sigma_C^2/n_A n_B n_C\}^{1/2}.$$

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This arises because the error comes from all three sources—between animals, between blocks within animals, and between cultures within blocks—and there are altogether $n_A n_B$ blocks and $n_A n_B n_C$ cultures.

Two problems remain once we know this formula: to estimate the variances so that it may be used in a particular case, and to use it to estimate the required sample sizes. To estimate the variances we need to have knowledge of a previous experiment or we must conduct a pilot experiment. The analysis of variance for nested or hierarchical designs (see e.g. Snedecor & Cochran, 1967, section 17.11) allows us to find the estimates we need. This can conveniently be carried out using GLIM (Baker & Nelder, 1978).

Now we must decide on the number of samples to be taken at each stage. Let us consider some particular values for the variances: suppose

$$\sigma_A^2 = 100, \quad \sigma_B^2 = 50 \quad \text{and} \quad \sigma_C^2 = 20.$$

With twenty animals available, and the technical ability to take ten blocks from each and set up five cultures per block, the minimum possible standard error is $(100/20 + 50/200 + 20/1000)^{1/2}$, or 2·30, but nearly all of this (2·24) is due to variation between animals; in other words it is hardly necessary to replicate the blocks or cultures at all, except to make sure that the experiment is technically successful. To put it another way, if this standard error is too large for some anticipated comparison between treatments, only increasing the number of animals involved will improve it. (We are aware that a better description of the sampling stages would have been 'animals', 'cultures' and 'sections' but for the purpose of this article the straightforward alphabetical labelling given by 'animals', 'blocks' and 'cultures' was thought to be worthwhile.)

This is a very widely applicable point: counting vast numbers of cells, or otherwise replicating experiments at the most deeply nested level, will not compensate for too small a group of basic experimental units, be they animal or human. Still less, of course, can anything compensate for an atypical sample at any level.

Note that if the experimental design has other features, such as that 'block i' from each animal is always from the same site, or that 'culture j' from each block is in the same culture medium, the analysis should take these into account. Snedecor & Cochran (1967) give many examples of how to estimate the components of variance for different experimental designs.

Example 2

Failure to observe a hierarchical sampling structure by treating all observations as statistically independent leads to erroneous estimates of standard errors, and hence to invalid significance tests. Consider the following example where six sections of tissue were subjected to one treatment and five to another. The labelling indices of the sections were estimated, but because of technical considerations different numbers of cells were sampled in different sections. Results are shown in Table 1.

What must *not* be done with these data is to ignore the fact that the sections are different and pool them to give that shown in Table 2. Table 2 gives a χ^2 value of 4·92 with 1 degree of freedom, and apparently a significantly higher labelling index under treatment 2; but it is quite wrong as the variability between sections has been ignored, and it is this with which the variability between treatments must be compared. The total number of cells counted is not relevant.

To compare the mean labelling index between the two treatments we should properly calculate the weighted means, weighting by the number of cells counted in each sample: this gives means and standard errors of 0·166 ± 0·025 (treatment 1) and 0·206 ± 0·020

Table 1. Experimental results

Treatment 1						
Section	1	2	3	4	5	6
Cells counted	200	100	200	150	150	200
Cells labelled	39	8	27	41	28	23
Treatment 2						
Section	7	8	9	10	11	
Cells counted	300	150	200	100	150	
Cells labelled	62	39	28	26	30	

Table 2. A wrong analysis

	Labelled	Unlabelled	Total
Treatment 1	166	834	1000
Treatment 2	185	715	900
Total	351	1549	1900

(treatment 2), and the difference is easily shown by a *t*-test to be non-significant. Snedecor & Cochran (1967, section 17.5) give the correct method of calculating the standard errors. Again GLIM provides a convenient method of analysis, and the Appendix contains a program to analyse the data. In this instance, ignoring the fact that different numbers of cells were counted per section makes little difference: comparison of the six labelling indices of treatment 1 with the five of treatment 2 via an ordinary two-sample *t*-test gives virtually the same answer as the weighted analysis. It is the pooling of data from the different sections which is more dangerous: this often leads to spurious significant results, sometimes to the loss of a properly significant one.

APPENDIX

Much of the data collected in cell kinetic experiments is amenable to analysis by GLIM, a package for the interactive analysis of 'generalized linear models' written by Baker and Nelder, and distributed by the Numerical Algorithms Group for the Royal Statistical Society. This journal has recently published what will surely be the first of many articles to use GLIM to analyse cell kinetic data (Camplejohn *et al.*, 1984). Below is shown a GLIM program to analyse the data of example 2 of the present article. The conclusion is similar to that of the weighted analysis, a *t*-value of 1·15 resulting.

```
$UNITS 11   $FACTOR TREATMENT 2
$DATA TREATMENT TOTALCELLS LABELLED $
$READ
  1 200 39   1 100  8   1 200 27   1 150 41   1 150 28   1 200 23
  2 300 62   2 150 39   2 200 28   2 100 26   2 150 30
$YVARIATE LABELLED   $ERROR BINOMIAL TOTALCELLS $
$FIT: TREATMENT $
$STOP
```

ACKNOWLEDGMENT

I would like to thank Dr J. P. Sunter for his comments on the articles in this series.

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Cell Tissue Kinet. (1984) **17**, 657–659.

Aspects of statistics in studies of cell proliferation

II. Standard errors of estimated parameters

2

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(*Received* 2 *September* 1983; *revision accepted* 29 *December* 1983)

Abstract. Statistical methods such as regression analysis are often used to estimate cell kinetic parameters, such as cell birth rate or tumour growth rate. The standard errors which the statistical procedures provide may be considerable underestimates of the true uncertainty surrounding the parameter of interest.

Simple linear regression of the mitotic index, or a function of the mitotic index, against time is used to estimate the cell birth rate from a stathmokinetic experiment. This works reasonably satisfactorily, but if turnover time is estimated from the birth rate the result is highly model-dependent—a fact not indicated by the standard error of the estimate. Likewise, growth rate estimates found by fitting curves to tumour growth data may depend critically on the curve chosen, and again this will not be indicated by the standard errors.

Example 1

The following set of data refer to a small stathmokinetic experiment (see e.g. Wright & Appleton, 1980) in which animals were killed at 0·5, 1, 1·5, 2 and 2·5 hr after administration of vincristine (see Table 1).

Regression of x_1 on t gives a cell birth rate (k_B) of 28·1 cells/1000 cells/hr with a standard error of 2·1 cells/1000 cells/hr; regression of x_2 on t gives 26·9 $\pm$ 2·0 cells/1000 cells/hr. The difference between the estimates is not large compared to the standard error of an estimate, so the result is relatively insensitive to the model postulated—in this case whether the age distribution of cells is rectangular or exponential. However the expression for the turnover time is $1/k_B$ for a rectangular distribution, and $(\ln 2)/k_B$ for an exponential age distribution, giving either 35·6 or 25·8 hr. The standard errors of these estimates may be found approximately from the formula:

$$\mathrm{SE}(c/z) = (|c|/z^2)\,\mathrm{SE}(z),$$

Table 1. Stathmokinetic data

Time after vincristine (hr)	t	0·5	1	1·5	2	2·5
Mitotic index	x_1	0·0149	0·0320	0·0462	0·0536	0·0744
$\ln(1 + x_1)$	x_2	0·0148	0·0315	0·0452	0·0522	0·0718

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where c is a constant and z is an estimated quantity. The assumption of a rectangular age distribution thus leads to an estimated apparent cell-cycle time of 35·6 ± 2·7 hr while an exponential age distribution gives 25·8 ± 1·9 hr. In other words the standard error fails completely to take into account any uncertainty about the cell age distribution, knowledge of which is crucial. Notice that it is quite wrong to assume that the age distribution is exponential because this leads to the smaller standard error.

Example 2

The lesson of the previous example applies very widely; as a further example we shall consider the estimation of the specific growth rate of a tumour from measurements of its weight (see e.g. Gratton, Appleton & Alwiswasy, 1978). Leaving aside all considerations of whether the weight of a tumour is a valid meausrement of its growth in cell kinetic terms—a question we shall return to—let us see how we may estimate the specific growth rate at 7 days from the data in Table 2.

Table 2. Tumour growth data

Time (days)	t	1	2	3	4	5	6	7
Weight (mg)	w	9·4	17·1	28·0	75·6	151	178	257
Time (days)	t	8	9	10	11	12	13	14
Weight (mg)	w	325	553	595	1080	1200	1220	1800

If we fit the popular Gompertz curve $\ln w = a - b\exp(-ct)$ the specific growth rate at time t is $bc\exp(-ct)$, whereas if we fit the logistic curve $\ln w = a/\{1 + b\exp(-ct)\}$ the specific growth rate at time t is $abc\exp(-ct)/\{1 + b\exp(-ct)\}^2$. A good curve-fitting program will enable standard errors for such quantities to be produced from the standard errors of (and covariances between) the estimates of a, b and c.

The fitted Gompertz curve is $\ln w = 8{\cdot}751 - 7{\cdot}452\exp(-0{\cdot}1226t)$ and the specific growth rate at 7 days is 0·387 ± 0·013 g/g/day. The fitted logistic curve is $\ln w = 7{\cdot}565/\{1 + 2{\cdot}941\exp(-0{\cdot}3066t)\}$ and the specific growth rate at 7 days is 0·442 ± 0·020 g/g/day. Once again the two analytical models give results which differ by considerably more than their standard errors.

DISCUSSION

In example 1 the turnover time which we were attempting to estimate was considerably longer than the duration of the experiment. It is therefore intuitively reasonable that our estimate should be dependent on something other than the experimental results themselves, namely the method of analysing these results. This is not the case in the second example: the tumour doubles in size several times and one may feel that the doubling time, and even more so the growth rate, should be easily estimable. The difficulty is that the growth rate does not remain constant, and the information available at different times throughout the experiment can only be of collective value if we use a particular model to link it all together.

The problem we are having in each case may be thought of in terms of accuracy and precision or, as they are sometimes called, validity and reliability. The methods we are using to estimate parameters give, by providing standard errors, indications of their reliability; they cannot consider their own validity. Thus, the model which is used, like the variable which is

measured, any systematic error in the measuring process, and the sampling method adopted, may all introduce unquantifiable bias, inaccuracy, invalidity (an unwelcome attribute, call it what you will) into the experiment. It is often very difficult to quantify this bias, but it is only sensible to see, by performing alternative analyses, whether the analysis of the data is introducing any. If the different analyses give answers which differ by more than the order of a standard error we may be sure that bias is a problem. Nor can we wish it away in comparative studies, because the bias in two instances may be quite different; for example if a treatment alters the cell age distribution from exponential to rectangular without our being able to detect the change, a comparison of the turnover times with and without treatment would be subject to bias. Likewise if a treatment alters the weight of a tumour without altering its cell number, any experiment using tumour weight as a measurement of cell number will be biased.

In conclusion, one should be very careful that one is estimating relevant parameters, and one should be suspicious of the standard errors of these estimates, as they may not quantify the real errors. Whenever possible it is advisable to analyse the data using alternative models to test the sensitivity to the estimation procedure of any result of that procedure.

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Cell Tissue Kinet. (1985) **18**, 111–113.

Aspects of statistics in studies of cell proliferation

III. Alternative models give different results

3

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(*Received* 12 *September* 1983; *revision accepted* 29 *December* 1983)

Abstract. Alternative models for the analysis of the spatial distribution of cells in an intestinal crypt cross-section are discussed. It is shown that different, though reasonable, models lead to different conclusions.

In the previous article in this series (Appleton, 1984) we discussed how an inappropriate analytical model of an experiment could cause the results, though reliable, to be invalid. Since this point is so fundamental, and since the building of alternative models to investigate their properties is so neglected, this article continues to look at different aspects of the same problem. We shall take one particular set of data due to Sawicki, Blaton & Pindor (1977) and show how analysing it in different, though basically similar, ways leads to different conclusions regarding the spatial arrangement of labelled cells, and hence the mode of migration of all cells, in the intestinal crypt. The derivations of the formulae which we shall use are not particularly straightforward and have not been presented here. Nevertheless it is hoped that the reader will accept the formulae and follow the main argument without being sidetracked into the algebraic details.

Sawicki and his co-workers studied cross-sections of crypts and measured the angular positions, relative to the axis of the crypt, of all labelled cells. If there are m labelled cells their relative positions may be defined by the m angles θ_i $(1 < i < m)$ between adjacent pairs. We may then define the statistic:

$$x = (1/m) \sum (\theta_i - 2\pi/m)^2,$$

which will take large values if labelled cells tend to group together and small values if they tend to arrange themselves at maximum distances apart. This is not the only way to analyse such data, and Potten *et al.* (1982) take a different approach, but it is a useful method on which to base this discussion.

To use the statistic x we must first know what its expected value is if cells are arranged at random round the circumference of a crypt cross-section, and in order to test the null hypothesis that they are indeed so arranged we must know something about its sampling distribution as well. Sawicki *et al.* (1977) modelled the crypt circumference as a circle and assumed that labelled cells were represented by points on the circle. If these points are randomly distributed on the circle the expected value of x is:

$$\{(m-1)/(m+1)\}\ (2\pi/m)^2.$$

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By treating separately the cases $m = 2, 3, 4, 5$ and finding in each case the observed value of x for a large number of crypt cross-sections they showed that the observed value was consistently and statistically significantly less than the expected value.

There are other models, of increasing complexity, which could be applied: firstly the variance of x may be calculated theoretically under the null hypothesis, and, as Sawicki *et al.* (1977) point out, this theoretical variance is larger than that observed, so that their results would be less significant. Secondly the crypt cells may be modelled as taking up a finite space on the circumference of the circle, so that if there are k of them the angle between the centres of adjacent cells is $2\pi/k$. (Alternatively this model is that the centres of the cells are at the vertices of a regular k-sided polygon.) This reduces the expected value of x to:

$$\{(k-m)/k\}\,\{(m-1)/(m+1)\}\,(2\pi/m)^2,$$

and if k is taken to be 16, the mean number of cells in their cross-sections, observed values are no longer significantly different from expected values for $m = 2, 3, 4$ or 5.

But this is just another model. We cannot say it is the right one. Suppose that the cells of interest are a different size from the others: proliferating cells may be smaller, for example, than mature cells. Does this make a difference? We need a further model to investigate this aspect. In fact if the labelled cells have radius $1 - \varepsilon$ times that of other cells the expected value of x is:

$$\{k(k-m)/(k-m\varepsilon)^2\}\,\{(m-1)/(m+1)\}\,(2\pi/m)^2,$$

larger than that of the previous model. And again the crypt circumference may be better modelled by an ellipse than a circle. Does that matter? Yes, it will again raise the expected value of x.

Table 1 shows the values of x observed by Sawicki *et al.* (1977), and the expected values $E(x)$ based on the following four models:

(i) cells represented by points on the circumference of a circle:

$$E(x) = \{(m-1)/(m+1)\}\,(2\pi/m)^2;$$

(ii) cells represented by k equal lengths of arc on the circumference of a circle ($k = 16$):

$$E(x) = \{(k-m)/k\}\,\{(m-1)/(m+1)\}\,(2\pi/m)^2;$$

(iii) cells represented by k lengths of arc on the circumference of a circle, labelled cells $1 - \varepsilon$ times as big as other cells ($k = 16$, $\varepsilon = 0{\cdot}25$):

$$E(x) = \{k(k-m)/(k-m\varepsilon)^2\}\,\{(m-1)/(m+1)\}\,(2\pi/m)^2;$$

Table 1. Mean value of the observed statistic x (from Sawicki *et al.* (1977), with angles measured in radians), and the expected values on the basis of four alternative models

Number of labelled cells	Observed value x	Expected value of x			
		Model (i)	Model (ii)	Model (iii)	Model (iv)
2	3·03	3·29	2·88	3·07	2·95
3	1·61	2·19	1·78	1·96	1·83
4	1·07	1·48	1·11	1·26	1·12
5	0·75	1·05	0·72	0·85	0·75

(iv) cells represented by k equal lengths of arc on the perimeter of an ellipse, eccentricity e:

$$E(x) = \{(k - m)/k\}\ \{(m - 1)/(m + 1)\}\ (2\pi/m)^2\ f(k, m, e),$$

where $f(k, m, e)$ is an unknown function of the total number of cells, the number of labelled cells, and the eccentricity of the ellipse; it must evaluated by simulation.

DISCUSSION

The reliability of the mean of the observed values of x is high. With the number of cells counted the standard error is of the order of 0·1. But different models change the expected values by considerably more than this. That is to say that the validity of any one of the models may be low. How are we then to interpret the results? In the case considered I believe we dare not. The most realistic model can only be a matter of judgement, unless more is known about the morphometrics of the system being investigated.

The analysis of most experiments involves explicit or implicit assumptions, which are sometimes critical to the interpretation of the data. Alternative models should be studied to see how critical such assumptions are.

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Cell Tissue Kinet. (1985) **18**, 133–135

Aspects of statistics in studies of cell proliferation

IV. Simulation

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4

(*Received* 12 *September* 1983; *revision accepted* 29 *December* 1983)

Abstract. Computer simulation of an experiment can help to reveal how effective that experiment might be in estimating cell kinetic parameters, or in elucidating the nature of a proliferative response to a perturbation of the steady state, such as a carcinogenic or therapeutic treatment. Writing a simulation also clarifies one's thoughts about possible mechanisms of the proliferative response.

CELLSIM (Donaghey, 1975) is a computer system for writing and running simulations of cell kinetic experiments. It is easy to model the killing of cells, blocking them in a particular phase of the cycle, labelling them, measuring their DNA content, etc. We can therefore perform simulated experiments on systems whose characteristics we know exactly and ascertain whether the experiments we are proposing to undertake can give us useful information about these characteristics.

Consider the following experiment. An experimenter wishes to measure the cell birth rate, using a stathmokinetic experiment, in the small intestine of control rats and rats given some treatment whose cytokinetic effect is known approximately: it is thought to kill some 40% of cells in S, block the entry to S for 6 hr, and then accelerate cells through G_1 by an unknown amount, but possibly for the next 24 hr reducing the cell cycle time from 11 to 9 hr. What would be the findings of a stathmokinetic experiment begun 12 hr after treatment? Figure 1 shows a CELLSIM program to investigate this. It is not necessary in order to understand the arguments of this article to follow the details of the program. Indeed it makes assumptions about the cell age distribution which could be challenged, but which are for the present unimportant; and some of it is concerned merely with how the results are printed. It is hoped however that the figure gives some impression of what is required to write a CELLSIM program.

It is necessary to replicate simulations many times, and in this case to take as the modelled experimental observations mitotic index data from different replicates, otherwise there will be serial correlation in the simulated data not present in the observed data; that is, the observations on the simulated experiment are interdependent; for example, in a single run of the program the number of arrested mitoses cannot decrease, whereas in the real experiment, where each point represents a different animal, it can. This, like the impossibility of modelling every single cell individually, is an undoubted drawback of such simulations and must be carefully borne in mind by the serious user of CELLISM or any other modelling system.

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```
CELL TYPES              CON, TRT;
STATES (1,2)            G1, S, G2, M;
TIME IN STATES (1,2)    G1: TRI(1,2,6),      S: TRI(4,6,8),
                        G2: TRI(0·5,1,1·5),  M: TRI(0·5,1,1·5);
INOCULUM (1,2)          G1: 55 UNIF(0,3),    S: 110 UNIF(0,6),
                        G2: 18 UNIF(0,1),    M: 17 UNIF(0,1);

NUMBER OF GROUPS (1,2) = 200;

KILL (2) AT TIME =48  S: 0·4;
BLOCK EXIT (2) BETWEEN 48 AND 54  G1;
CHANGE TIME IN STATES (2) AT TIME =48  G1: TRI(0,1,2);

BLOCK EXIT (1,2) BETWEEN 60 AND END  M;

REPORTS 24 HOURS;
CHANGE REPORTS, AT TIME =47,  2 HOURS;
CHANGE REPORTS, AT TIME =59,  0·25 HOURS;

RANDOM NUMBER SEED =328511;
SIMULATE 62·5 HOURS
```

Fig. 1. A CELLSIM program to model the experiment described in the text.

Table 1 shows the birth rates from ten simulations, estimated by finding by least squares the slope of the mitotic accumulation line. It is apparent that the mean birth rate for the control experiment is close to that expected (91 cells/1000 cells/hr) for a steady-state system (rectangular age distribution) with a cell cycle time of 11 hr and all cells proliferating. The change after treatment to a cell cycle time of 9 hr has, however, not resulted in an increase to 111 cells/1000 cells/hr but an increase to 301 cells/1000 cells/hr. Thus the perturbation to the age distribution which the cytotoxic agent causes influences the birth rate estimate by far more than any change in cell cycle time can.

Table 1. Birth rates (cells/1000 cells/hr) in control and treated rats for ten simulations

											Mean
Control	85	76	113	73	90	91	89	79	84	87	87
Treated	295	309	306	292	313	306	310	296	289	298	301

If the experimenter were to proceed with the real experiment he would be doing so knowing that he could not detect changes in cell cycle time. Likewise he could carry out further simulations to see the effects of changing growth fraction, different assumptions about the cell killing and blocking effects of the cytotoxic agent, rescheduling the stathmokinetic experiment, etc. All this, of course, takes time—both the experiment's and the computer's—but potentially it can save one from carrying out experiments, the results of which are virtually uninterpretable. Alternatively, by placing the real results in the context of simulated ones, it may enable one to interpret otherwise uninterpretable results, though this desirable outcome is frequently found to be a rather optimistic hope rather than a likely occurrence.

Aherne & Dunnill (1982) describe a simulation of an experiment using stereology to estimate blood-vessel size, in which they create a physical model of the system they wish to study. They test their analytical method on this model and find it to be satisfactory. Computer simulation is very similar: instead of a physical model of a static structure we may construct a computer model of a kinetic process, but the object is still to test the applicability of an

analytical method (probably based on rather simple assumptions which we suspect to be violated by the system we wish to study) to the elucidation of real data. Further details of the use of this technique in the context of cell proliferation may be found in another expository article by the present author (Appleton, 1983).

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Biomathematics and Cell Kinetics
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THE MEASUREMENT OF TUMOUR GROWTH RATES

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ABSTRACT

Data were obtained on the growth of a transplantable fibrosarcoma in a number of mice. Several equations were used to fit the data and have been compared in terms of their ability to estimate the specific growth rate of the tumour and its ultimate size. Marked differences were found between the estimates obtained although all the curves could be considered to fit the data adequately.

INTRODUCTION

The estimation of tumour growth rates from measurements of tumour weight or volume plays an important part in the description of the kinetics of a tumour. It may, for example, be used in conjunction with estimation of birth rates from stathmokinetic experiments to try to measure the cell loss rate, possibly the most useful single characterisation of the cell kinetics of a tumour.

Vieira and Hoffman[1] have compared the logistic and the gompertz functions in terms of their ability to fit growth data and conclude that both methods should be used and the better fit on the basis of residual variance should be accepted. Swan[2] also deals with these curves (without fitting data) and discusses the general form of curves which might be expected to fit tumour growth data adequately. We have fitted several different functions to the same data and have investigated in particular how the choice of function affects the estimate of growth rate at different stages of growth.

The gompertz and logistic functions tend towards limiting values as time passes. These values are in this case estimates of the ultimate tumour size, assuming it to reach an equilibrium where cell loss balances cell production; the values given by the different curves are compared.

MATERIALS AND METHODS

One hundred and forty-four female Balb/c mice aged 3 months were injected in either flank with a suspension of 10^6 tumour cells. The tumour was a fibrosarcoma which had undergone about 160 passages since arising spontaneously in a 5 month old castrate male Balb/c mouse (see Aherne, Camplejohn and Wright[3]). Animals were killed at times up to 17 days after implantation and the tumours were dissected free of skin, muscle and connective tissue, blotted dry and weighed. This study was part of a series of experiments to compare the growth characteristics of a

pair of tumours with those of a single tumour; in this paper only the results for tumours in the right flank will be presented. Very similar results hold for the tumours in the left flank whose growth has also been analysed.

Figure 1 shows the weights of all the tumours and the data are summarised in Figure 2 which gives the geometric mean weight for each day. It is the latter data, weighted for numbers of observations, which has been used in fitting the curves.

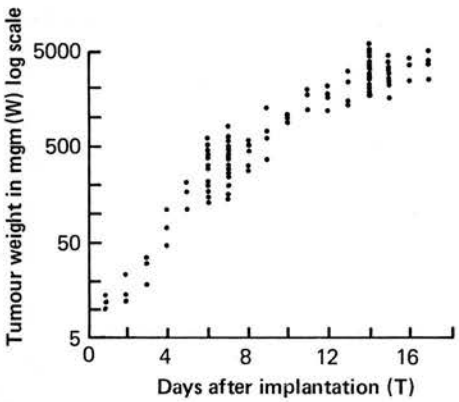

Figure 1. Tumour weight against days after implantation for 144 animals. (not all points are visible for days 7 and 14)

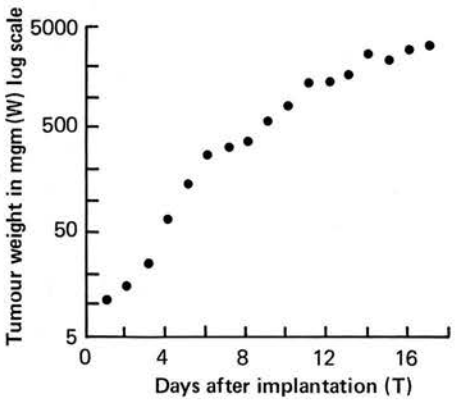

Figure 2. Average tumour weight (geometric mean) against days after implantation.

MATHEMATICAL EQUATIONS

Let $W_k(t)$ be the weight (in mgms) of the tumour in the k^{th} animal t days after implantation, and suppose that the relationship between weight and time can be expressed in the form

$$\log W_k(t) = f(t;\underline{\alpha}) + \varepsilon_k$$

for some function $f(t;\underline{\alpha})$ with parameters $\underline{\alpha} = (\alpha_1,\alpha_2,\ldots,\alpha_p)$ where the ε_k are independently normally distributed with zero mean and variance σ^2. (Inspection of Figure 1 shows this to be a reasonable assumption.) It is then appropriate to use the method of least squares to fit the response function $f(t;\underline{\alpha})$ to the observations $\log W_k(t)$.

It is possible to obtain not only estimates $\hat{\underline{\alpha}}$ of the parameters $\underline{\alpha}$ but also an estimate of σ^2 and hence of the standard errors of $\underline{\alpha}$ (see for example Gallant[4]). We may then find an approximation to the standard error of the estimate of a function $g(t;\underline{\alpha})$ of the parameters from

$$\mathrm{var}\{g(t;\hat{\underline{\alpha}})\} \simeq \sum_{i=1}^{p}\sum_{j=1}^{p} \mathrm{cov}(\hat{\alpha}_i,\hat{\alpha}_j)\frac{\partial g}{\partial \alpha_i}\frac{\partial g}{\partial \alpha_j}$$

Seven functions $f(t;\underline{\alpha})$, all with 3 parameters, have been used; these are listed in Table 1. The parameters for each have been designated a, b and c with the convention that for the data fitted all the estimates $\hat{a}$, $\hat{b}$ and $\hat{c}$ are positive. The specific growth rate is $\frac{dw}{dt}/W$, that is $\frac{df}{dt}$, and the expressions for this quantity are also shown in Table 1. The functions chosen have different characteristics: functions 1-4 rise with time to approach asymptotic values, so that they are in that sense more reasonable curves to fit than the rational or quadratic curves which are functions 5-7. However, the use of curve-fitting as a technique can be justified empirically for any curve that is capable of providing good estimates of the parameter sought so it is legitimate to investigate the performance of any curve over the range of observations. Functions 1 and 2, 3 and 4, 5 and 6 are related in pairs: if one of a pair fits

$$\log W_k(t) = f(t;\underline{\alpha}) + \varepsilon_k$$

the other fits

$$\log W_k(t) = \log f(t;\underline{\alpha}) + \varepsilon_k$$

Notice also that the gompertz and logistic functions are closely related, for if $y = a\text{-}be^{-ct}$ then $1/y = A/\{1-\exp(B-ct)\}$ where $A=1/a$ and $B=\log b/a$.

RESULTS

The fitted curves are shown in Figure 3. None is an obvious bad fit to the data, although the pattern of residuals for curve 3 is slightly suspect. In terms of the residual variance curve 2 is best, closely followed by curve 1 (see Table 1); that is, the curves of gompertz type are better than the others.

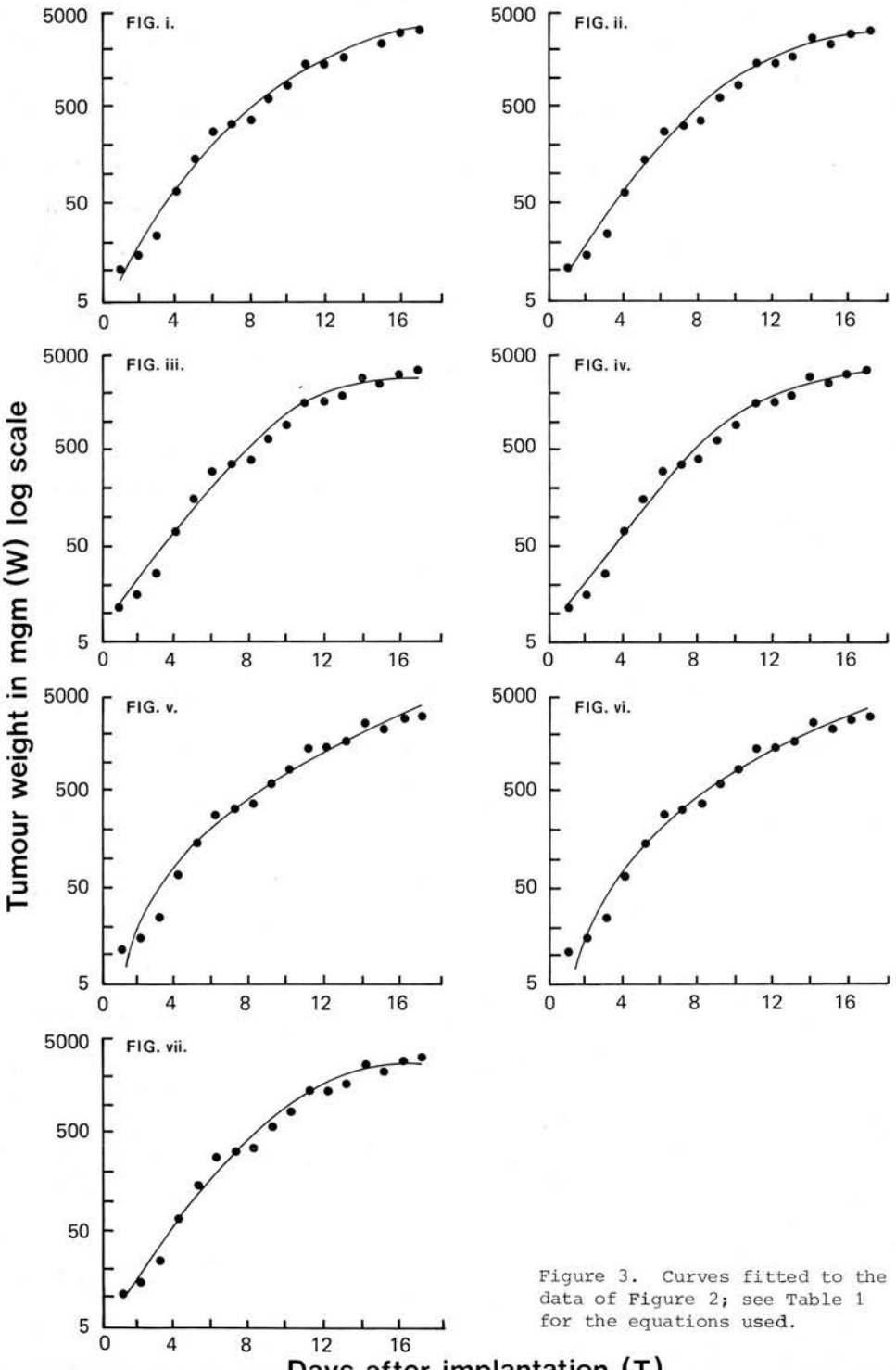

Figure 3. Curves fitted to the data of Figure 2; see Table 1 for the equations used.

TABLE 1

Curve Number	Curve type	Response function $f(t;a,b,c)$	Specific growth rate $g(t;a,b,c)$	Residual Variance
1	Gompertz	$a-be^{-ct}$	bce^{-ct}	0.24
2	Gompertz	$\exp(a-be^{-ct})$	$bce^{-ct}\exp(a-be^{-ct})$	0.24
		Write $z=\exp(b-ct)$		
3	Logistic	$\log\{\frac{a}{1+z}\}$	$\frac{cz}{1+z}$	0.35
4	Logistic	$\frac{a}{1+z}$	$\frac{caz}{(1+z)^2}$	0.29
5	Rational	$\log\{\frac{t+at^2}{b-ct}\}$	$\frac{1+2at}{t+at^2}+\frac{c}{b-ct}$	0.40
6	Rational	$\frac{t+at^2}{b+ct}$	$\frac{1+2at}{b+ct}-\frac{c(t+at^2)}{(b+ct)^2}$	0.35
7	Quadratic	$a+bt-ct^2$	$b-2ct$	0.26

Curves fitted to tumour growth data, with expressions for specific growth rate.

TABLE 2

Curve fitted	Specific growth rate (mgm/mgm/day)			
	Day 3	Day 7	Day 10	Day 14
1	.64	.38	.26	.15
2	.61	.42	.26	.12
3	.53	.46	.29	.07
4	.54	.44	.27	.11
5	.64	.32	.25	.21
6	.72	.34	.25	.19
7	.58	.41	.29	.12
Mean	.61	.40	.27	.14

Estimates of the specific growth rate on 4 days given by fitting 7 different curves.

TABLE 3

Curve fitted	Weight (gms)	95% confidence limits		
1	4.96	3.0	-	8.3
2	2.90	2.1	-	4.0
3	1.84	1.5	-	2.2
4	2.38	1.8	-	3.1

Estimates of eventual tumour weight, with 95% confidence limits, given by fitting 4 different curves.

Table 2 shows the estimates of specific growth rate at 4 different days for the 7 curves. At day 10 the estimates are close enough for any to be useful, but at other times they show considerable disparity. The coefficient of variation of the 7 estimates are 10, 13, 6 and 35 percent at 3, 7, 10 and 14 days respectively.

The approximate standard error for the specific growth rate at 7 days using curve 1 is 0.01. This means that the estimate of the error of estimation which is obtained by assuming that this is the correct curve to fit is noticeably smaller than the error involved in fitting a different, though plausible, curve. Since none of the equations used have any sound biological foundations we would be ill-advised to treat the usual standard error estimates as good indications of the precision of the estimate of specific growth rate. It is likely, according to Gallant[5], that using the likelihood ratio is a better way of obtaining accurate confidence limits, and this is borne out by our own experience.

The estimates of the ultimate weight of the tumour given by the first 4 curves are shown in Table 3. They clearly differ considerably, to the extent that quite different conclusions could be reached about how nearly the tumour had approached its eventual size at a given time. The magnitude of the confidence interval for curve 1 in particular means that a comparison of eventual size between a single tumour and one of a pair of bilateral tumours could yield very little information. It is, of course, essential that the same form of equation be used to fit sets of data whose growth characteristics are to be compared.

DISCUSSION

Several other curves with three parameters and many more with more than 3 could have been fitted to the tumour growth data. Gratton[6] has used cubic splines in addition to the 3 parameter curves we have reported here. (A possible advantage of fitting splines is that the effect of a single point (x_i, y_i) on the function $f(x)$ decreases as $|x-x_i|$ increases.) We can also obtain a satisfactory fit using Mendelsohn's[7] (1963) suggestion which amounts to log wt = a log (bx+c). These investigations strengthen our conviction that a great many curves may be used to give apparently reasonable representations of the data but when the equations are used to estimate the specific growth rate of the tumour the results may be misleading and the standard error found by the curve fitting method may lead to a considerable underestimate of the possible error.

Since a similar situation arises in stathmokinetic experiments, where the standard error is calculated on the assumption of linearity of the collection function over the duration of the experiment, which may not be true because of a delay in action of the stathmokinetic agent or death of arrested metaphases, the use of tumour growth data to calculate the growth rate and stathmokinetic experiments to calculate the cell birth rate may lead to very erroneous estimates of the cell loss rate. The difficulty is aggravated by the fact that the growth

rate in mgm/mgm/day is assumed to be convertible into cells/cell/day.

On the basis of our results, however, we can suggest that the least erroneous results are likely to arise if the growth curve fitted has the form

$$\log W(t) = \exp(a-be^{-ct})$$

and confidence intervals for the specific growth rate are calculated using the likelihood ratio.

ACKNOWLEDGEMENT

We would like to thank Miss P. Barratt for typing this article and Mrs. D.M. Tomkinson for preparing the illustrations.

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POSTSCRIPT

We hope that three aspects of this paper will be seen to have general applicability to the use of mathematics in cell kinetics. Firstly, we have based our arguments on data; we feel that a mathematical model, however elegant, which begins with arbitrary assumptions not suggested by data and which leads to irrelevant conclusions not tested against data, is an arid exercise of little advantage to biology. Secondly, even if a model is constructed on sound biological principles and its predictions are shown to agree well with the observations it was designed to simulate, there is no guarantee that the mechanisms of the model are mimicking those of the biological system. A geocentric model of the solar system using circles and epicycles for planetary motion is in many respects a satisfactory representation of reality. Nevertheless it is wrong. Models may be used to discredit a hypothesis; they cannot verify one. We agree that we have (purposely) not constructed any models in this paper,

but if data can be fitted by such diverse functions as we have used it is not an improbable inference that observation will often not be able to distinguish between alternative models. Finally, we have pointed out that it is necessary to quantify the probable errors of estimation involved in determining the value of any particular parameter of a model. If good theoretical error estimates cannot be obtained (and it is usually possible to build up a likelihood surface numerically) the precision of the parameter estimates should at least be assessed qualitatively by investigating the adequacy of the fit obtained using non-optimal values of the parameters.

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The robustness of methods of analysing stathmokinetic experiments

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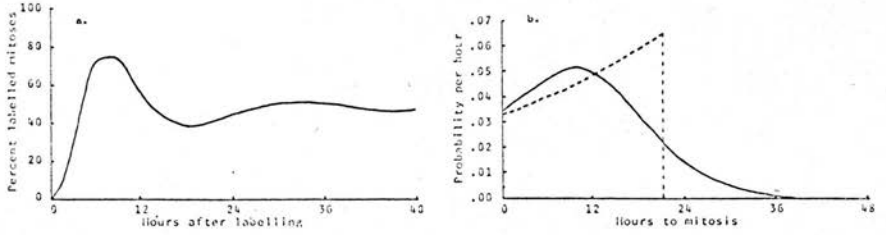
Introduction.

Estimates of cell cycle times are often calculated from the rate of accumulation of mitotic cells after the administration of an agent which arrests cells in mitosis. It is the purpose of this paper to describe briefly an investigation of the accuracy of these estimates with particular reference to three factors, namely the conditions of growth of the cell population, the distribution of cell cycle times and the method of fitting equations to the data points.

Method of investigation.

The language CELLSIM (Donaghey 1973) was used to conduct a simulation of a stathmokinetic experiment. Cells were initially given an exponential age distribution consistent with constant phase times of: G_1 6 hours, S 10 h, G_2 4 h and M 1 h; and the population was allowed to grow for 80 hours with each cell producing 2 daughters at mitosis and with phase times Erlangian distributed with mean times as above. All work was done for the Erlangian parameter (n) taking values 1, 2, 3, 4 and 5. For brevity and simplicity we shall report here only the results for n = 3. This gives a coefficient of variation for the cell cycle time of 34 %. The expected FLM curve, produced by a slightly modified version of the program due to Gilbert (1972), and the distribution of duration to mitosis which these conditions produce are shown in the figure.



a) PLM curve and b) distribution of times to mitosis for an exponentially growing population. Phase times are Erlangian distributed with parameter 3, and means for G_1 S, G_2 and M are 6, 10, 4 and 1 hours respectively. The dotted line is for an exponentially growing population with constant phase as above.

After 80 hours simulated time, cells were blocked in mitosis and the mitotic index (I_m) was recorded every 15 minutes for 2¾ hours (12 readings).

Fifty replicate simulations were carried out with different random numbers and, from these, 20 sets of results were constructed for which successive readings were not correlated. In other words, the experiment simulated was one in which each data point was provided by a different piece of tissue.

Results.

The usual method of calculation of the mean cell cycle time (T_c) is to fit the equation (Cleaver 1967)

$$\log_2 (1 + I_m) = \frac{1}{T_c} (t_m + t)$$

to the observed data by the method of least squares. From the 20 simulations we obtained estimates of T_c with mean 19.1 hours and standard deviation 2.4 h.

Distribution of cell cycle times.

The value obtained for T_c is significantly lower than the correct value of 21 hours. Although the estimate of the mean rate of entry of cells into mitosis is unbiased, T_c will in general be underestimated because it is calculated as the reciprocal of a harmonic mean which is less than the arithmetic mean.

Method of curve fitting.

Using the 50 simulations the variance of I_m at each of the 12 data points can be shown to be (to a good approximation) proportional to I_m itself.

It is therefore appropriate to minimise a weighted sum of squared residuals. This procedure still gives a mean T_c of 19.1 hours but reduces the standard deviation of the 20 estimates to 2.0 h.

Growth conditions.

So far the analyses have assumed (correctly) that growth is exponential. If we erroneously assumed that steady state conditions prevail we would fit the curve (Cleaver 1967)

$$I_m = \frac{1}{T_c} (t_m + t)$$

This gives us an estimate of 25.5 hours using the weighted least squares method. In fact, fitting this line to 12 points which are exactly on the curve given by exponential growth with a cell cycle time of 21 hours, gives an

estimate of 28 hours for T_c with a negligible standard error. This indicates that confidence limits for estimates of T_c given by the method of least squares as applied to stathmokinetic data are likely to be considerably too narrow.

Conclusions.

In analysing the results of a stathmokinetic experiment large errors in the estimate of mean cell cycle time may be made if the growth conditions assumed do not in fact occur. Furthermore, even if the correct growth conditions are used, the estimate of mean cell cycle time will be biased towards low values, although the mean rate of entry into mitosis is unbiased. It is better to use weighted least squares than unweighted least squares to reduce the variance of the estimates.

We are aware that there are also practical difficulties in the use of stathmokinetic agents : a delay before the drug takes effect or death of mitotic cells near the end of the experiment both lead to overestimates of the cell cycle time.

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ACKNOWLEDGEMENT

One of the authors (P.W.) is supported by a grant from the North of England Council of the Cancer Research Campaign, which is gratefully acknowledged.

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Cell Tissue Kinet. (1980) **13**, 643–663

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THE METAPHASE ARREST TECHNIQUE

A CRITICAL REVIEW

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(*Received* 31 *January* 1979; *revision accepted* 10 *December* 1979)

ABSTRACT

The accuracy and precision of the stathmokinetic experiment for the determination of cell birth rate are discussed from practical and theoretical viewpoints. Topics covered include the determination of the optimal dosage of the metaphase arresting agent, the times at which observations should be taken and which cells should be counted, the correct method of estimating cell birth rate and the dependence of any derived estimate of the turnover time on the age distribution of cycling cells. Failure to consider any of these points can lead to considerable inaccuracy. It is further shown that the stathmokinetic method is a rather imprecise one for measuring the turnover time and the duration of mitosis.

Data are present comparing the results of using the technique with those obtained from autoradiographic studies.

A criticism of classical cell kinetic techniques, and particularly of those which involve autoradiography, is their time-consuming and laborious nature. Furthermore, many kinetic responses are ephemeral and occur far too quickly to permit application of, for example, the fraction of labelled mitoses (FLM) method for the calculation of cell cycle phase durations and proliferative rates. In such situations some investigators rely on measurements of two proliferative indices, the mitotic index (I_m) and the labelling index (I_s) measured autoradiographically after exposure of the tissue to tritiated thymidine ([^{3}H]TdR) for 1 hr. It is well known, however, that these indices reflect not only changes in the rate of cell proliferation brought about by alterations in the cell cycle time or the proportion of proliferating cells, but also changes in phase durations, *viz*., the mitotic duration (t_m) and the duration of DNA synthesis (t_s), respectively.

For these reasons, some workers have turned to another method, the metaphase arrest or stathmokinetic technique. Correctly applied, this provides a useful kinetic parameter, the rate of entry of cells into mitosis, or the cell birth rate, sometimes called the mitotic rate. From the cell birth rate, one can calculate the cell cycle time if all cells are proliferating or, if there is a non-proliferating population, one may estimate the potential doubling time in tumours or the

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turnover time in, say, epithelial tissues. The method can give results over as short a period as 2·5 hr and involves the study of paraffin sections only.

The earlier literature in this field was summarized by Eigsti & Dustin (1955) and in 1967 Tannock emphasized some of the requirements for suitable stathmokinetic agents, requirements which were best met by the periwinkle alkaloid vincristine. However, in addition to using an agent with adequate metaphase arresting properties, there are several critical experimental points which must be observed and, even if the experiment has been carried out well, problems are encountered in the analysis of the data.

It is the purpose of this review to identify these problems in experimental technique and in data analysis. Moreover, there are numerous instances in the recent literature where the method has been incorrectly applied and inappropriate measurements obtained which are often dignified by the term 'mitotic rate'; it is clearly important for the future of this method that these abuses be identified. There is increasing use of the method in clinical situations, notably for measuring the cell birth rate in human tumours (Refsum & Berdal, 1967; Camplejohn, Bone & Aherne, 1973; Iversen *et al.*, 1974; Wright *et al.*, 1977; Taylor, Iversen & Bjerknes, 1977) and, if maximum information is to be gained from these investigations, then it is crucial that the scope and limitations of the method are appreciated. Faulty application has brought the metaphase arrest technique into disrepute; its precision when correctly applied is only moderate and, when incorrectly used, it is potentially misleading.

Metaphase arrest agents

There are many compounds which have been shown to have metaphase arresting properties; of these the earliest used was colchicine, now replaced by its less toxic derivative demecolcin (N-desacetyl-N-methylcolchicine)—more usually called colcemid. More recently the periwinkle alkaloids vincristine and vinblastine have been used as stathmokinetic agents. The arresting action of these compounds depends upon an interaction with the mitotic spindle or its subunits, which results in the failure of assembly or disruption of the mitotic spindle, with the production of characteristic abnormal mitotic figures (*cf.* Wilson *et al.*, 1974). These arrested metaphases have been called 'colchicine metaphases' (Clarke, 1971) and are usually readily distinguishable from normal metaphases.

Investigators have varied in their choice of stathmokinetic agent; of those workers who have compared the metaphase arresting properties of several agents, Tannock (1967) and Smith, Thomas & Riches (1974) agreed that vincristine is superior, while colcemid proved to be the worst agent of all. However, Clarke (1971) and Nome (1975) have produced evidence, discussed below, that colcemid can be useful in several tissues.

Metaphase arrest techniques

Basically, the method hinges upon the measurement of the slope of the line which describes the accumulation of arrested metaphases. The results of a typical experiment are presented in Fig. 1, which shows the accumulation of arrested metaphases in the crypts of the normal human small intestine. Vincristine sulphate was injected intravenously at time 0 and biopsies were taken at the times indicated. The measurements of the slope of the line gives an estimate of the birth rate, mitotic rate or the rate of entry into mitosis.

It is convenient to approach the correct use of the technique in terms of a series of nine questions.

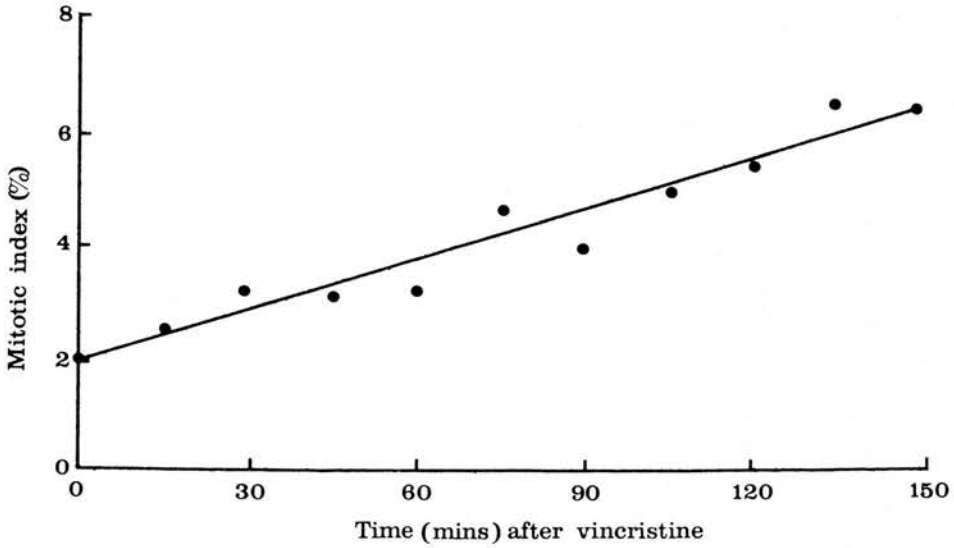

FIG. 1. Mitotic index accumulation data for normal human jejunal mucosa after an intravenous injection of vincristine (0·045 mg/kg body weight), followed by serial biopsy with the Quinton multiple biopsy tube (from Wright *et al.*, 1973).

What dosage should be used? It is of considerable importance to determine the optimal dosage of the stathmokinetic agents for the tissue to be studied; too few authors, when describing stathmokinetic experiments, have shown dose–response curves for the agent and tissue used. This point is specially relevant, since Tannock (1967) and Nome (1975) have shown that optimal dosages for the various agents may differ in the same tissue and that different tissues may have different optimal dosages for the same agent. Tannock (1967) analysed the characteristics of dose–response curves of metaphase arrest agents. Figure 2a shows such a curve for vincristine in the epidermis of patients with psoriasis (Duffill *et al.*, 1977); vincristine, in the doses shown, was injected intradermally into stable psoriatic plaques and biopsies were taken 2·5 hr after injection. To begin with, an increase in dose caused the metaphase index to increase; this is attributed by Tannock to the arrest of an increasing proportion of metaphases, but is also probably due to the disappearance of any delay period before metaphase arrest is complete since, at least *in vitro*, this delay period is dose-dependent (Taylor, 1965; see below). Further increase in the dose led to degenerating, pyknotic metaphases being predominant and hence there was not only a loss of arrested metaphases but an actual decrease in the arrested metaphases as a proportion of the total number of cells. High doses of these agents appear to be toxic and to lead to rapid metaphase degeneration. Fitzgerald & Brehaut (1970) also showed depression of metaphase indices in lymphocytes *in vitro*, with increasing doses of colchicine. In the dose–response curve shown for vincristine in psoriatic epidermis, the possibility that the degeneration effect begins before the arresting effect is complete cannot be excluded; this implies lowering of the peak and underestimation of the mitotic index. The degree of underestimation may vary with the time after administration, which makes the calculation of the cell birth rate difficult.

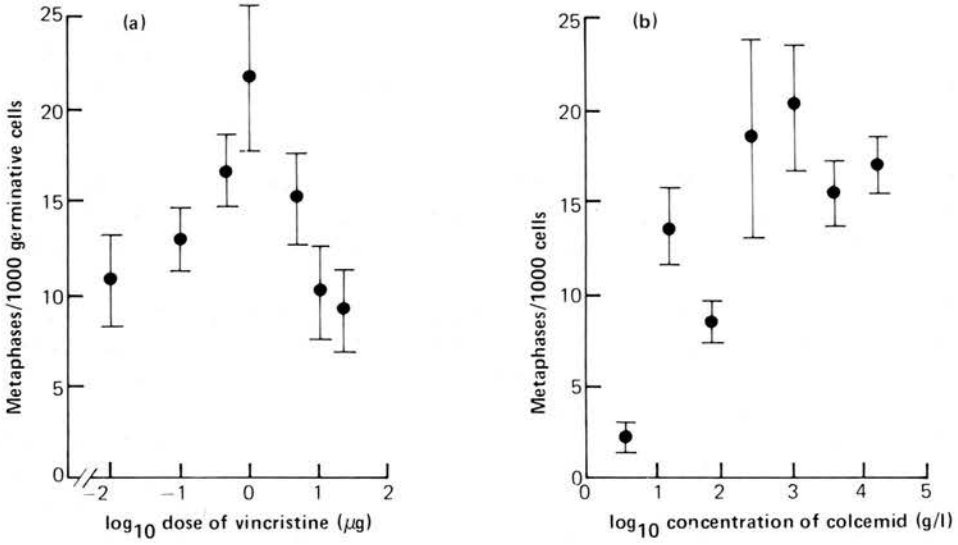

FIG. 2. (a) Dose–response curve for vincristine in human psoriatic epidermis *in vivo*. Vincristine was injected intradermally in the doses indicated into the periphery of stable psoriatic plaques and biopsies taken 2·5 hr later under local anaesthetic. Vertical bars represent standard errors for the points (from Duffill *et al.*, 1977). (b) Dose–response curve for colcemid in the mouse coagulating gland *in vitro*. Explants were incubated with colcemid in the doses shown, and readings were made 4 hr later. Vertical bars represent standard errors for the points (from Morley, 1970).

A dose–response curve for colcemid, which exhibits different features, is shown in Fig. 2b. The results are for primary explants of the mouse coagulating gland in organ culture and the readings were taken 4 hr after the introduction of colcemid in the concentrations shown (Morley, 1970). The curve rose to a value which remained more or less constant over the dose range used. The increase in metaphase index is again due to the increase in the proportion of metaphases arrested and this is supported by measurements of the proportions of cells in the different mitotic phases for increasing doses of colcemid: after the plateau is attained anaphases are no longer present. In this instance over the concentration used, metaphase degeneration has not caused a reduction in the maximum index. Assuming that there is a critical concentration below which no metaphase degeneration occurs, this concentration is higher than the minimum concentration for maximum metaphase arrest.

In rat small bowel, Tannock (1967) obtained dose–response curves of the type shown in Fig. 2b for colchicine, vinblastine and vincristine; and in tumour isografts for colchicine and vincristine only, colcemid and vinblastine showing curves similar to those in Fig. 2a. Tannock's results with colcemid have been criticized by Clarke (1971), because of the small numbers of animals used and because the within animal variability was not given, and also by Nome (1975), who considered that Tannock's collection period of 4 hr was too long to be optimal for colcemid. In a study of the effects of colcemid and vinblastine on the rat small bowel crypt, Clarke (1971) showed a decreasing number of arrested metaphases as the dose increased from 1 to 5 mg/kg, with both agents, over a period of 2·5 hr; the larger doses increased the number of overtly degenerate metaphases. With colcemid, however, variation in metaphase collection and degeneration was less apparent. Furthermore, Nome (1975) also

obtained a broad-peaked dose–response curve for colcemid in several tissues. Smith *et al.* (1974) concluded that, over the dose range 1–4 mg/kg in a transplantable mouse carcinoma, colcemid showed no variation with dose over a 2 hr period, nor vincrinstine over a 4 hr period.

Thus, although Tannock (1967) considered that colcemid exhibited poor dose–response characteristics, the results given in Fig. 2b for the mouse coagulating gland and those of Clarke (1971), Smith *et al.* (1974) and Nome (1975) indicate that, under certain experimental conditions, colcemid may be used.

A further criterion of an optimal dose or concentration is completeness of metaphase arrest, i.e., the absence of anaphase escape. If metaphase arrest is not complete the birth rate will be underestimated; therefore, it is essential that anaphase escape be excluded for the concentration of an agent to be considered adequate. For example, Clarke (1971) reported anaphase escape in small bowel crypts of the rat with doses of colcemid below 2·5 mg/kg. Recently, Riches *et al.* (1977) have shown that doses of vincristine, which produce complete arrest in maternal tissues, show anaphase escape in foetal tissues; whether this is due to differential sensitivity of foetal tissues or to difficulties with transplacental passage is not yet clear. In this respect, Williams (1973) reported that although metaphase arrest was complete in foetal gut and brain, increasing doses of colcemid or vinblastine gave diminishing metaphase indices; this was considered to reflect an embryospecific inhibitory effect on cells in DNA synthesis.

This leads to a further consideration; an important requirement of the optimal dose is that it should not affect the flux of cells through other phases of the cell cycle; in this context Fitzgerald & Brehaut (1970), studying cultured lymphocytes, reported that large doses of colchicine depressed DNA synthetic rate and flux through G_2, although Hell & Cox (1963) showed that this effect was less marked with the same dose of colcemid in epidermis. On the other hand, Clarke (1971) concluded, from his comparison of tritiated thymidine and stathmokinetic agents, that metaphase blocking agents at suitable doses do not effect the rate of entry of cells into mitosis, in the small bowel. Also in the small intestine, Jellinghaus, Schultze & Maurer (1977) have reported that vincristine (in usual doses) does not affect the flux of cells into or out of DNA synthesis, as measured by double labelling methods. Ideally, such effects should be monitored for each experimental system, though this has seldom been done.

In conclusion, one should, if at all possible, determine the dose-response characteristics of the select agent for the tissue to be studied; a suitable curve will show a plateau value, and the optimal dose or concentration is the lowest that will arrest all metaphases and prevent anaphase escape over the experimental period selected, *for that specific tissue*: once this is decided, then other factors, such as delay period, linearity of metaphase collection and any metaphase degeneration, can be assessed for that dosage (see below). However, as with any *in vivo* procedure which necessitates injection, the administration of a stathmokinetic agent is potentially stressful, and could lead to release of catechol amines and adrenocortical steroids, both of which are active in inhibiting proliferative activity.

Which cells should be counted? Having ensured that metaphase arrest is complete, there remains the problem of what to count. There will be prophases and arrested metaphases in the samples and some workers have counted both of these (e.g., Wright, Morley & Appleton, 1972) whereas others have counted metaphases only (Smith *et al.*, 1974). One disadvantage of the first procedure is that criteria for the recognition of prophases vary and reproducibility

between observers may not be as good as for the second method, although a separate count of prophases does allow a check to be made on any changes in the flux into or duration of prophase during the experimental period. Either procedure will be satisfactory for estimating the cell birth rate, but a point of practical importance arises in the calculation of mitotic duration. It will be shown below that mitotic duration can be calculated from the ratio of the native mitotic index to the mitotic rate. If only metaphases are included in the native mitotic index, then only the duration of metaphase will be estimated; if, however, all phases of mitosis are included, a value for the total mitotic duration will be derived.

The number of arrested metaphases should be related to the total cell population of which they are part; there has been a tendency for investigators, particularly in the study of squamous epithelium, to relate the mitotic or metaphase count to a unit length of basement membrane and not to a total cell count (e.g., Bullough & Laurence, 1968; Bertsch *et al.*, 1976). This necessitates expression of the cell birth rate as, e.g., cells produced per unit length of epidermis and comparison of birth rates between experiments involve the important assumption that nuclear population density remains constant. If certain experimental conditions produce an increase in nuclear density, the cell production rate per unit length of epidermis may rise, even if the birth rate in cells/cell per hr is unchanged.

Some investigators have increased the population assessed by counting all metaphases in a large measured area, and then computing the total nuclear population in this area on the basis of sample counts in smaller areas; this may be helpful in instances where metaphase indices are particularly low, as in adult mouse lung (Simnett & Heppleston, 1966). Smith *et al.* (1974) used a modified technique employing a point counting system to measure the tumour cell component of tumour isografts and computed the number of tumour cells and metaphases per unit volume; this allows calculation of the cell production rate for tumour cells only.

The intestinal crypts are closed systems from which arrested metaphases are unable to escape during the experiment. Microdissection and counting of crypts and arrested metaphases allows the calculation of the cell production rate per crypt (Wimber & Lamerton, 1963; Clarke, 1971). If the turnover time is to be calculated, then these metaphases must be expressed as a fraction of the total cell population, so the mean number of cells per crypt must also be calculated. An advantage of this method is in avoiding the problems which accrue from differences in the diameter of arrested metaphases and interphase nuclei or in their apparent diameter, relative to the plane of section.

Correction factors have been used to compensate for the observation that arrested metaphases differ from associated interphase nuclei in size, at least in tissue sections; for example, Simnett (1968) considered that metaphases arrested by colcemid, with chromosomes spread by exposing the tissue to hypotonic saline, were larger than interphase nuclei and calculated a correction factor of 0·8 by which the observed metaphase index should be multiplied. In tumour isografts, Smith *et al.* (1974) showed that metaphases arrested by vincristine (measured at an unspecified time after vincristine) had effectively the same diameter as interphase nuclei. Clarke (1968, 1970) concluded that, in embryonic chick duodenum, colcemid arrested metaphases were smaller than interphase nuclei and suggested a correction factor of 1·54. These correction factors were assumed to be constant with time, but Tannock (1967) proposed that although a newly arrested metaphase is larger than a normal interphase nucleus, with advancing age they decrease in size and these cell size considerations tend to cancel themselves out. However, it is possible that either the initial enlargement or the later

contraction of arrested metaphases may outweigh each other; the problem is amenable to experimental exploration in any system.

In the intestinal crypts mitotic cells tend to be nearer the axis of the crypt than are interphase cells. Longitudinal sections may therefore include mitoses from columns of the crypt other than those whose interphase cells are to be counted, and an overestimate of the mitotic index will result. Tannock (1967) gives a method of obtaining a correction factor in this situation, and workers should be on their guard, especially in structured tissues, against similar biases.

Care must also be taken when counting cells in any heterogeneous tissue: if a tumour of radius 1 cm has a non-proliferative centre of radius 0·5 cm the cell production rate of the tumour is 88% of the production rate of the outer portion, assuming constant nuclear density. The constituent parts of a tissue should therefore be sampled in proportion to their volume if an unbiased overall rate is to be calculated or else the sampling fractions should be taken into account in the analysis. It may indeed often be unrealistic to attempt to describe the proliferative activity of a heterogeneous tissue by a single cell birth rate; but if this is to be done it should be noticed that the numbers of cells to be counted, which will depend on the error we are prepared to accept, cannot be determined purely on the basis of binomial counting error: variability between sections may well be much more important than variability within sections.

When should readings be taken? As mentioned above, the stathmokinetic method depends on the measurement of the slope of the metaphase accumulation line, so it is critically important to establish linearity of metaphase collection. In recent years this has all too often been disregarded. A very common error is to take only one reading, 3 or 4 hr after injection of the stathmokinetic agent, and call this the mitotic rate (Bertalanffy, 1964; Bullough & Laurence, 1968, 1970; Hennings & Elgjo, 1970; Laurence & Randers Hansen, 1972; Clausen, 1975; Argyris, 1977) or mitotic index (Simnett & Heppleston, 1966; Chopra, 1974). Others have divided this single reading by the collection period and calculated an hourly 'mitotic rate' (Elgjo, 1967; Hennings & Elgjo, 1970; Simnett, 1972; Tvermyr, 1972; Iversen *et al.*, 1976; Bertsch *et al.*, 1976). But suppose we have taken a single reading, 4 hr after injection of a stathmokinetic agent, in two tissues in which the birth rate must be compared. Fig. 3a shows a single reading in one of the tissues; obviously, such a single reading would be compatible with many different lines, including both of those illustrated, which give quite different estimates of the birth rate. Consequently, any comparison with another tissue would not be in terms of birth rates since we have not measured them: it is clearly possible to obtain the same single 4 hr reading when the birth rates are markedly different. Implicit in the single reading method is an assumption that there is linearity of metaphase collection, and that the line extrapolates to the origin. Detailed analyses of metaphase collection in intestine (Wright *et al.*, 1972; Al-Dewachi *et al.*, 1975a) and in squamous epithelium (Nome, 1975) show that very frequently this is not so. The method is often used to monitor ephemeral responses, in situations where the native mitotic index is changing rapidly (e.g., after various perturbations); this will compound the error and results can be most misleading (Clausen, 1975; Bertsch *et al.*, 1976).

In another common procedure two readings are taken: the native (time zero) mitotic index and another 3 or 4 hr later. This is often done in human investigations where, for ethical reasons, there is a limit to the number of biopsies which can be taken (Iversen, 1967; Taylor *et al.*, 1977; Wright *et al.*, 1977). There are tacit assumptions here too; that the agent

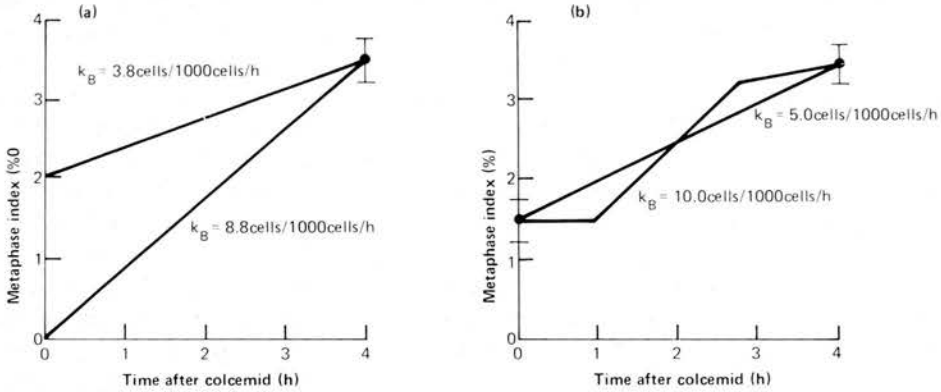

FIG. 3. (a) A possible error which can ensue from using a single reading of the mitotic index after injection of a stathmokinetic agent. The single point is consistent with both lines which give very different estimates of the birth rate, k_B. (b) When two points are available large errors can still be made because of the possibility of a delay period or metaphase degeneration. Both the straight line and the curve, which give quite different estimates of k_B, are possible.

becomes active immediately after injection and that metaphase collection occurs in a linear manner thereafter. Figure 3b shows schematically what happens when this is not so and Fig. 4 is an example; the tissue is mouse oesophagus and the agent vincristine in a dose of 1 mg/kg. If only the readings at 0 and 4 hr were used, the birth rate would be underestimated, because there is a delay period before metaphase arrest becomes effective, in this case some 75–90 min after injection.

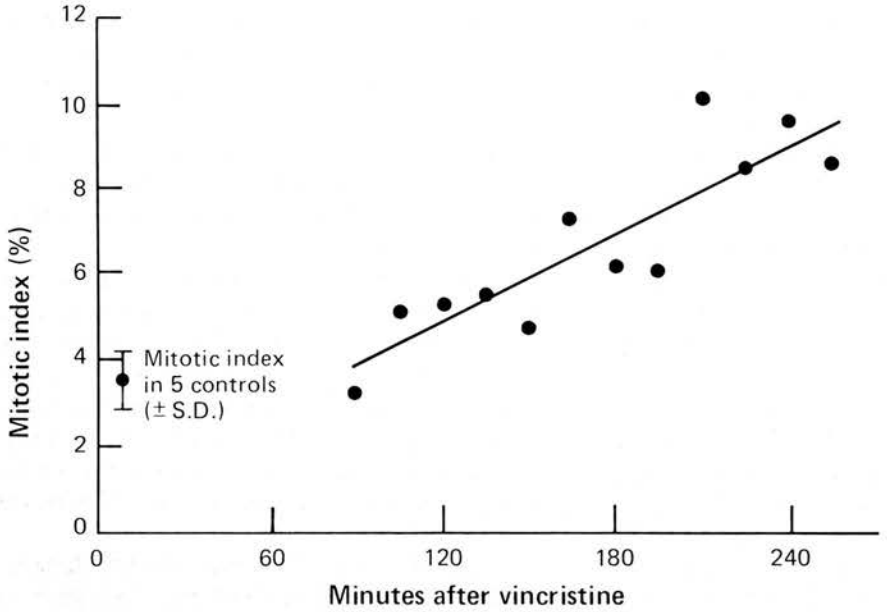

FIG. 4. Mitotic index accumulation data for mouse oesophagus squamous epithelium after 1 mg/kg vincristine injection. Note the delay period before accumulation begins (from Appleton *et al.*, 1977).

This phenomenon of a delay period before metaphase arrest is complete is well recognized *in vitro*, where it has been shown to be related to concentration, decreasing in duration with increasing dose. Taylor (1965) showed that the time of appearance of the first arrested metaphase in a human cell line decreased with dose of colchicine to about 1 hr over a range of doses from 5 to 25 l/mol $\times$ 10^{-8}; at still higher concentrations the duration of the lag approached zero. This indicated that cells having completed different stages in mitosis are differentially sensitive to colchicine and also that, at higher concentrations, cells in metaphase when the agent is administered can be arrested very quickly. Using [^{3}H]colchicine, Taylor was able to show that the rate of binding to microtubular protein was proportional to concentration; similarly Inoue (1972), in *Chactopterus*, and Gaulden & Karlson (1951), in grasshopper neuroblasts, showed that increased concentrations of colchicine reduced the time for the spindle to disappear. However, Puck & Steffen (1963) could not confirm this in their *in vitro* work with colcemid, where only cells entering mitosis after colcemid application were arrested.

In mammalian cells the same considerations may apply and the kinetics of arrest appear to differ in different tissues; for example, there is a delay period of about 20 min in mouse jejunal mucosa with vincristine at a dose of 1 mg/kg (Al-Dewachi *et al.*, 1975a,b), whereas no such delay occurs in rat jejunum under the same experimental conditions. Moreover, Nome (1975) showed that optimal doses of colcemid produced delay periods in mouse epidermis and colon, but not in jejunum or squamous forestomach. However, Elgjo (cited by Taylor *et al.*, 1977) reported that in mouse epidermis the metaphase arrest line extrapolated back to the origin; this may indicate that there was a delay period equal to the duration of mitosis, and a similar situation occurred in the studies of Brown & Berry (1968) in their analysis of hamster cheek pouch epithelium. It cannot be assumed that this is always the case: none of Nome's (1975) metaphase collection lines, for several mouse tissues, extrapolate to the origin, and neither does the line for mouse oesophagus shown in Fig. 4. Of course, if metaphase arrest was immediate, the line would extrapolate back not to the origin or the native mitotic index, but to the native metaphase index; the data in Fig. 4 would be consistent with immediate onset of arrest and a metaphase index of about 1%. However, from the *in vitro* studies of Taylor (1965), the delay period was dose-dependent (see above). In this situation it would appear that the decreasing delay period was due to more rapid arrest of metaphases with increasing dose; there is, as yet, no evidence that the delay period is dose-dependent *in vivo* and the reason for the delay period must await further work.

A further claim made by Taylor *et al.* (1977) was that the native mitotic index was 'almost always higher' than initial counts after colcemid. Taylor (1965) has analysed in detail the kinetics of arrest during the first hour of colchicine action *in vitro*; at concentrations where no delay period was evident there was no initial fall in mitotic index but neither was there when a delay period was evident. It is clear that it is not possible to generalize; the behaviour of the mitotic index at early time periods is a reflection of the individual agent, its concentration or dose and the tissue being studied. Route of administration is also important, since metaphase arrest after intravenous injection of colchicine is established before arrest, after subcutaneous or intraperitoneal injection (Stevens Hooper, 1961).

Consequently, it is crucial in animal experiments to investigate whether there is a delay period and if so to take it into account when calculating the birth rate. In human work it is essential to appreciate the assumptions one is making when one has only two readings.

What is the optimal collection period? Tannock (1967) considered that the cell birth rate

should be measured over the linear portion of the collection line; he demonstrated linearity for different periods with different agents; with vincristine in the rat small bowel linearity was obtained until 4 hr after injection. However, Frei *et al.* (1964) claimed that vincristine showed linear accumulation for 12 hr and Smith *et al.* (1974) reported a 10-hr linear collection, also with vincristine, while Taylor (1965) obtained 11 hr linearity with colchicine *in vitro*. It is necessary to view these results with caution, because metaphase degeneration will probably occur to some extent in all experiments (at least on mammalian cells) lasting more than 3 hr; for example, Clarke (1971) noted a considerable amount of metaphase degeneration only 3·5 hr after injection of colcemid (2·5 mg/kg) in rat jejunum, while Morris (1967) reported degeneration occurring after 3 hr in mouse duodenum and after 2.5 hr in thymus. However, Shackney, Bunn & Ford (1976) considered that degeneration occurred only after 8 hr in mouse bone marrow.

In a mathematical analysis of the stathmokinetic technique, Trucco (1965), while appreciating that metaphases do degenerate over 'relatively short periods of time', assumed no loss of metaphases. Puck, Sanders & Petersen (1964) corrected their counts of arrested metaphases for degeneration in CHO cells in culture. The *in vivo* problem has been analysed by Aherne & Camplejohn (1972), by repeating the experiment of Puck *et al.* (1964) for the mouse small intestine: Fig. 5 summarizes their results. By giving an injection of tritiated

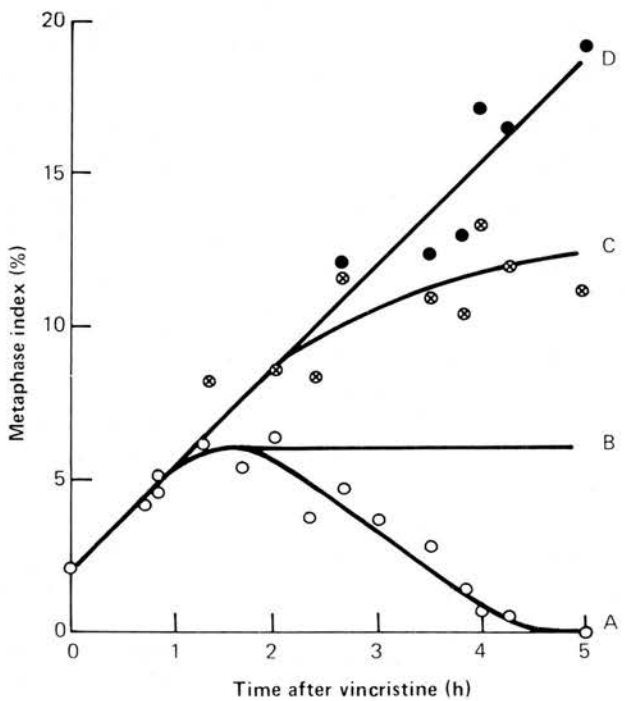

FIG. 5. Metaphase degeneration in the mouse jejunal mucosa. Vincristine (1 mg/kg) and [^{3}H]TdR (1μCi/g) were injected at time zero. Line A (○) shows the proportion of all cells which are unlabelled metaphases, line B is the plateau to be expected if these did not degenerate; line C (⊗) is the proportion consisting of all metaphases, and line D (●) is formed by correcting line C for the degeneration of line A (from Aherne & Camplejohn, 1972).

thymidine at the same time as vincristine, the fate of unlabelled arrested metaphases (which were in G_2 at the time of injection) can be followed. If degeneration did not occur, then the unlabelled G_2 cohort of metaphases would remain constant in number over the duration of the experimental period, as suggested by curve B. In fact, after 2 hr the G_2 metaphases decreased in number and had largely disappeared by 5 hr after vincristine (curve A), indicating that all metaphases in G_2 at the time of injection had disappeared. Hence, it is necessary to correct the observed mitotic index (curve C) for metaphase loss after 2 hr; assuming that metaphase loss was random, Aherne & Camplejohn (1972) derived a correction function by which the observed metaphase index was multiplied and curve D was the result.

We must therefore be suspicious of the results of Fisher (1968), who used arrest periods as long as 12 hr in human epidermis without proof of linearity and it is also reasonable to ask if the 10 hr linear collection claimed by Smith *et al.* (1974) could not be subject to a systematic error after a relatively short period, which would decrease the estimate of the birth rate.

There is also some evidence that the rate of metaphase degeneration is dose-dependent Tannock (1967) and Clarke (1971) both noticed increased numbers of degenerate forms with higher doses of agents, particularly of vinblastine.

Ideally, one should investigate the kinetics of metaphase degeneration, as one should investigate a delay period, in each tissue to be studied and only take readings in the period before metaphases begin to degenerate. Because of this consideration, several workers have confined their observations to relatively short periods, for example, 2·5 hr in the small bowel (Clarke, 1970; Al-Dewachi *et al.*, 1977). This may be reasonable when the cell cycle time is not too long in relation to the arrest period, but if longer arrest periods are contemplated because the cell cycle time is itself longer, then it is necessary to explore metaphase degeneration in some detail.

Having collected the data, how is the line fitted? When the problems of adequate dosage, the delay period and metaphase degeneration are solved or avoided, there remains the problem of the best way to analyse the data. Many authors have merely applied linear regression analysis to the untransformed data and duly calculated the slope of the line (e.g., Camplejohn *et al.*, 1973; Smith *et al.*, 1974). It is well to consider the several factors which give rise to the constants in the equation of any curve we may fit. One such consideration is the age distribution of the cell population under study.

First a word about nomenclature. Some confusion has occurred concerning the use of the term 'cell cycle time' as measured by stathmokinetic experiments; it is a cell cycle time only if all cells are proliferating and the growth fraction is unity. Consequently, the cell cycle time can be calculated only if the growth fraction is known, but if there are no data about the growth fraction the parameter measured has variously been called the potential doubling time or turnover time; more thought should be given to using the cell birth rate itself rather than converting it to one of these hypothetical times.

If all cells are cycling and the age distribution is rectangular, then the correct equation to fit to the mitotic or metaphase index, $M(t)$, data is the straight line

$$M(t) = \frac{t_m + t}{T_c} \qquad (1)$$

where the constants t_m and T_c are the mitotic duration and cell cycle time. If, however, the age

distribution is known to be exponential, we should use the collection function of Puck & Steffen (1963) and fit

$$\log_{10}[1 + \mathrm{M}(t)] = \frac{\log_{10} 2}{T_c}(t_m + t) \tag{2}$$

or more simply

$$\log_2[1 + \mathrm{M}(t)] = \frac{t_m + t}{T_c} \tag{3}$$

It is also possible to treat populations with intermediate age distributions; consider a population of cells maintaining a constant size, by cell birth and migration. At each mitosis two new cells are produced, of which $1 + p$ remain in the population ($0 < p < 1$). The remainder leave the population. Supposing all cells in the population to cycle, the probability $P(\tau)$ that a cell will be of age τ is

$$P(\tau) = \frac{1 + p}{p} \frac{\log_e(1 + p)}{T_c} \exp\left[\frac{-\log_e(1 + p)}{T_c} \tau\right] \tag{4}$$

(Steel, 1968; Appleton, Wright & Dyson, 1977) and we should fit

$$\log_{1+p}\{1 + p\mathrm{M}(t)\} = \frac{t_m + t}{T_c} \tag{5}$$

(Duffill *et al.*, 1977). It is also possible, using much the same argument, to introduce information about the growth fraction; operationally, however, this is unimportant, since accurate knowledge of the growth fraction implies the existence of detailed [^{3}H]TdR studies, in which case the metaphase arrest method is unlikely to be employed. While it is important to realize that the curve fitting procedure depends in principle upon the format of the age distribution, it is not necessarily of practical importance when estimating the cell birth rate (Duffill *et al.*, 1977). However, the age distribution should be remembered in one practical situation; when the age distribution can be measured or calculated it is worthwhile incorporating this into the curve fitting, because derived values such as the turnover time and, where calculation is possible, the cell cycle time depends upon it. Instances where this is possible include the mouse jejunal crypt; Schultze *et al.* (1972) have shown that fluxes into and out of S are equal for the whole crypt and thus a rectangular age distribution may be appropriate. However, in crypt bases the flux into S exceeds that out of S (Burholt, Schultze & Maurer, 1976) which would be expected on the basis of an exponential age distribution; indeed, such an age distribution was used by Tutton (1973) in the analysis of his stathmokinetic data for the 10 basal cell positions of the rat jejunal crypt.

In layered tissues such as stratified squamous epithelium, Appleton *et al.* (1977) have shown that the age distribution can be calculated from measurements of the direction of the mitotic axis; in squamous epithelia with a single layer of proliferating cells, and all mitotic axes in the plane of the basal layer (e.g., hamster cheek pouch, Brown & Berry, 1968), the age distribution will be exponential. If all axes are vertical with respect to the basement membrane (e.g., embryonic mouse epidermis, Smart, 1970) the age distribution is rectangular. In most adult squamous epithelia, including human epidermis, the mitotic axis can be in either

direction and the age distribution for the basal layer is intermediate between exponential and rectangular, but can be calculated. In systems with multiple layers of proliferating cells (for example, human psoriatic epidermis, plant meristems), the age distribution for the proliferative tissue as a whole can be shown to be very nearly exponential, independent of the direction of the mitotic axis, but for individual layers it need not even be intermediate between rectangular and exponential.

In rapidly proliferating transplantable tumours in exponential growth it is reasonable to assume an exponential type age distribution. However, in older tumours, where cell loss is significant, not independent of the age of the cell, and the growth fraction is low, the age distribution may be of exponential type, but does not fall from a factor of two from the beginning to the end of the cell cycle and a rectangular age distribution may be more appropriate (Camplejohn *et al.*, 1973). Indeed, if cell death is principally at mitosis and is leading to regression, even a rectangular age distribution may be a poor approximation. This may be especially relevant for studies of human tumours.

Duffill *et al.* (1977) showed that the choice of the correct age distribution in stathmokinetic experiments was very important when estimating the turnover time, though not so necessary for the birth rate. For example, in an experiment with vincristine in human psoriatic epidermis, using a rectangular age distribution gave a mean value for the birth rate of 0·013 cells/cell per hr, while application of the (correct) exponential age distribution gave 0·012 cells/cell per hr, but these provide mean values for the turnover time of 79 and 56 hr, respectively. Similarly, in mouse oesophageal basal cells (Fig. 4), mean birth rate estimates of 0·020 and 0.019 cells/cell per hr were obtained with rectangular and exponential age distributions, equivalent to turnover times of 49 and 36 hr, respectively. The correct age distribution, as calculated from the direction of the mitotic axes, has $p = 0{\cdot}37$ and using equation 5 to fit the line gave a birth rate of 0·020 cells/cell per hr and a turnover time of 43 hr. It is never possible to tell from the data which age distribution should be used: if the mitotic index $M(t)$ appears to be linearly related to time t then so do all functions of the form $\log\{1 + pM(t)\}$.

In summary, we should stress that age distribution considerations are only important when a derived value such as the turnover time is needed from stathmokinetic data; the birth rate is not nearly so dependent on the age distribution and is therefore much more useful.

A further problem in analysing the data is the statistical method used to fit the line; most workers have used an unweighted least squares technique (Clarke, 1971; Tutton, 1973; Camplejohn *et al.*, 1973; Smith *et al.*, 1974). Since the variance of the mitotic (or metaphase) index, after for example, vincristine, is usually proportional to the mitotic index itself, Whittall, Appleton & Wright (1975) considered it appropriate to minimize a weighted sum of squared residuals and found that this procedure was likely to reduce the variance of the turnover time estimate obtained.

One problem which has received insufficient attention is the measurement of birth rates in situations where the native mitotic index is itself increasing. This is a very common situation in cell kinetic studies and occurs during the recovery of tissues from perturbation, e.g., in renewal systems after irradiation and in instances of induced DNA synthesis such as liver regeneration. If a metaphase arrest experiment is carried out during such a proliferative response, the rate of entry into mitosis will not be constant over the experimental period, but will increase with time. Fig. 6a shows the changes in the native mitotic index in the jejunal crypts of rats refed after 4 days starvation (Al-Dewachi *et al.*, 1975b). Fig. 6b shows the

results of a metaphase arrest experiment with vincristine (1 mg/kg) carried out 16 hr after refeeding, fitted by equation 1. Since the mitotic index is itself increasing then, theoretically, the curve fitted should not be a straight line, and Fig. 6c shows the result of fitting a quadratic expression to the data on the assumption that the native mitotic index was itself increasing

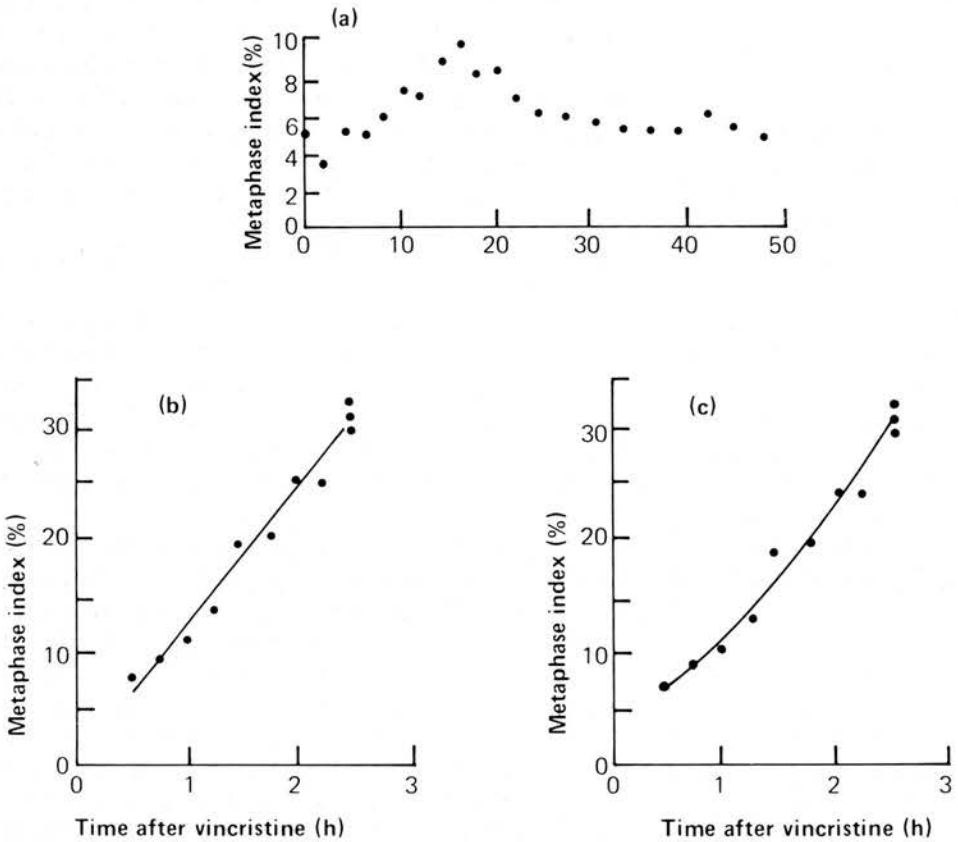

FIG. 6. Metaphase accumulation when the native mitotic index is itself increasing. (a) The mitotic index in the jejunal crypts of rats refed after 4 days starvation. (b) The results of a metaphase accumulation experiment with vincristine (1 mg/kg) 16 hr after refeeding, the line being fitted assuming a constant rectangular age distribution. A quadratic equation fitted to the same data; since the native mitotic index is changing with time the cell birth rate must be too, and it can be calculated approximately from the tangent to the curve. (From Al-Dewachi *et al.*, 1975b.)

linearly. The mitotic rate at any time may be found from the tangent to the curve. Notice that once again the stathmokinetic data themselves are not sufficiently accurate to provide evidence of non-linearity.

In some normal tissues the mitotic index is not constant but changes diurnally, Møller, Hartmann & Farber (1978) have used the metaphase arrest method to monitor this periodicity in the hamster cheek pouch by hourly metaphase collections with colcemid.

What sort of precision is obtained? Having fitted the appropriate equation to the data and

obtained estimates of the birth rate and the turnover time, it is necessary to quantify the precision of the estimates. This is best done by calculating their 95% confidence intervals, that for the birth rate being derived directly from the least squares curve-fitting process.

For the basal cells of the mouse oesophagus (Fig. 4) the birth rate has been shown to be 0·020 cells/cell per hr; 95% confidence limits for this value are 0·013 and 0·027 cells/cell per hr. The estimate of the turnover time, which is 43 hr, has a 95% confidence interval stretching from 32 to 67 hr. Twelve readings were available for analysis in this experiment and the variability about the fitted line was fairly typical; the best possible dose of vincristine had been administered, the delay period was excluded from analysis and a reasonable estimate of the age distribution was used. Yet the precision is poor. It must be concluded that the stathmokinetic method is imprecise even when conditions are optimal and if the conditions we have been describing are not met even worse results must be expected. It is only because so few published results have quoted confidence limits that this has not been more obvious; of course when only one or two readings have been used it has not been possible to calculate even approximate confidence limits.

If there is more than a trivial amount of variability in cell cycle times, a correction factor is required to compensate for the fact that any derived turnover time, or cell cycle time, is biased towards the faster cycling cells; the estimate of the turnover time obtained is not the mean turnover time. Appleton *et al.* (1977) give a factor by which to multiply the estimate to reduce the bias; this amounts to dividing by $1 - CV^2$ where CV is the coefficient of variation of the cell cycle time. Unfortunately, this factor will seldom be known with any accuracy, but its relevance should be remembered.

How do stathmokinetic estimates compare with standard autoradiographic techniques? Table 1 shows some instances where we have measured the birth rates in the same tissue and kinetic situation with both stathmokinetic and autoradiographic methods (the fraction labelled mitoses method) using tritiated thymidine. In such a comparison it is critical that these criteria are met; a comparison such as that of Bertalanffy (1964), who compared tritiated thymidine and stathmokinetic results in different strains and ages of the same species, is not realistic remembering the considerable strain and age differences which have been described. Similarly Tannock (1967), in a comparison of the cell cycle time as measured by FLM and metaphase arrest techniques, compared measurements of jejunal mucosa in two different strains of rats; further, in a transplantable tumour, Tannock compared doubling time measurements from growth curves, and the potential doubling time from the metaphase arrest data; such a comparison makes the absolute assumption of no cell loss (Steel, Adams & Barrett, 1966), an unusual situation even in tumours growing exponentially.

It can be seen from Table 1 that, generally speaking, agreement is fairly good, and we can be reasonably certain that we are measuring the same variable using the two techniques. At the same time we should recall that, although the FLM method is widely regarded as the best available method for the measurement of the cell cycle time and its constituent phases, the technique also has its problems, notably the effect of cell synchrony imposed by diurnal variation in fluxes into and out of the several phases (e.g., Tvermyr, 1972).

Can the mitotic duration be estimated reliably using the metaphase arrest method? The mitotic duration can be measured only very roughly from FLM curves and so investigators have looked to the stathmokinetic technique for better estimates (Evensen, 1965); in the small bowel they have quoted values (Clarke, 1970; Wright *et al.*, 1972; Pelc, 1971; Tutton, 1973), often to the hundredth part of an hour and seldom with any estimate of precision.

TABLE 1. A comparison of estimates of cell birth rate (k_B) in various tissues, found from metaphase arrest and FLM experiments; k_B is in cells/1000 cells per hr

Tissue	Kinetic state	Metaphase arrest k_B	FLM k_B	Ref.
Mouse jejunum	Normal	52	49	1
Mouse jejunum	15 hr after ara–C	73	83	2
Rat jejunum	Normal	72	55	3
Rat jejunum	After 96 hr starvation	38	38	4
Rat jejunum	15 hr after hydroxyurea	63	62	5
Mouse descending colon	Normal	26	19	6
Mouse transverse colon	Normal	13	14	6
Mouse ascending colon	Normal	16	16	6
Mouse caecum	Normal	18	20	6
Mouse sarcoma	7 days after implantation	37	30	7
Mouse sarcoma	14 days after implantation	36	29	7
Mouse sarcoma	21 days after implantation	33	32	7

Notes (i) A rectangular age distribution was assumed for each tissue except the sarcoma for which an exponential distribution was used. (ii) The growth fraction was estimated in every case from the ratio of observed to theoretical labelling indices, the latter being calculated from phase durations derived from the FLM curve which was analysed by the method of Gilbert (1972). k_B values from the FLM experiments use these growth fractions; this was not always so in the original articles. 1. Al-Dewachi *et al.*, 1975a. 2. Al-Dewachi *et al.*, 1979. 3. Wright *et al.*, 1972; Al-Dewachi *et al.*, 1974. 4. Al-Dewachi *et al.*, 1975b. 5. Al-Dewachi *et al.*, 1977. 6. Sunter *et al.*, 1979. 7. Wright, 1975; Aherne *et al.*, 1977a,b.

From equation 1 we see that the accumulation curve, transformed thus into a straight line, meets the time axis at $t = -t_m$. If, however, there is a delay in the action of the stathmokinetic agent the estimate of t_m found in this way will be an underestimate by the (imprecisely known) length of the delay and it is better to estimate t_m as $M(O)/k_B$, i.e., the observed native mitotic index divided by the birth rate calculated from the accumulation line. For the data of Fig. 4, $M(O) = 0{\cdot}036 \pm 0{\cdot}0030$ (s.e.) and $k_B = 0{\cdot}020 \pm 0{\cdot}0035$, so that t_m is estimated as 1·8 hr. The standard error of a quotient can be determined approximately from the standard errors of the numerator and denominator:

$$\text{s.e.}(t_m) \simeq t_m \left\{ \left[\frac{\text{s.e.}(M(O))}{M(O)} \right]^2 + \left[\frac{\text{s.e.}(k_B)}{k_B} \right]^2 \right\}^{1/2}$$

In this case the standard error of the estimate of t_m is 0·35 hr so that the 95% confidence interval for t_m is about 1·1 to 2·5 hr, which is too wide to be very useful.

How useful is the method for measuring proliferative rated in human tissues? Because of the possible mutagenic properties of nuclear-bound tritiated thymidine and the supposedly limited numbers of biopsies which are needed for the stathmokinetic method, many workers have looked to the latter to provide information about proliferative rates in human tumours and in normal tissues. For example, Refsum & Berdal (1967) reported a mean potential doubling time (t_{pd}) of 2·6 days in a group of tumours, mainly of the mouth and throat, while Fulker, Cooper & Tanaka (1971) reported t_{pd} values in vesical carcinoma varying from 22·2 days in well-differentiated tumours to 2·2 days in undifferentiated carcinomas and considered

that there was a negative correlation between the degree of differentiation and t_{pd}. Aherne & Buck (1971) found a mean t_{pd} of 4·2 days in a group of five cases of neuroblastomas and 7.8 days for a similar group of nephroblastomas. In Burkitts lymphoma, Iversen *et al.* (1974) reported t_{pd} values ranging from 25 to 64 hr, using colcemid, and Taylor *et al.* (1977), with the Kaposi sarcoma, obtained t_{pd} estimates varying between 88 and 462 hr.

These results depend upon one or two readings after injection of the arresting agent and, as discussed above, there are numerous important assumptions made when using so few readings and it is not possible to get any idea of the worth of the data. On the other hand, Meyer & Donaldson (1969) measured t_{pd} in oral squamous carcinoma, using serial biopsies after vinblastine injection and found values varying between 6 and 9 days. Camplejohn *et al.* (1973) used small numbers of biopsies in nineteen patients with carcinoma of the rectum to produce a composite metaphase accumulation line, giving a t_{pd} of 192 hr, compared with 82 hr in the adjacent rectal mucosa. Wright *et al.* (1977) studied metaphase accumulation in human gastric carcinomas for varying periods after vincristine injection, taking up to nine biopsies at four time intervals, and compared cell production rates in the carcinomas with those in adjacent gastric mucosa; both in the carcinomas and in pyloric mucosa cell birth rate, estimates ranged from 3 to 24 cells/1000 cells per hr. There was great uncertainty in each estimate with lower 95% confidence limits of zero for the birth rate, not from any statistical considerations, but on the assumption that cells were indeed being born.

A complicating factor which must be remembered in human tumour studies is the problem of heterogeneity in proliferative rates between adjacent portions of the same tumour (Refsum & Berdal, 1967). A recent analysis by Aherne *et al.* (1977a) has shown that human rectal carcinomas and neuroblastomas show differences in mitotic index between different areas, which are of the same order as differences between individual tumours. This consideration will affect stathmokinetic studies and can only be overcome by taking large numbers of biopsies from different parts of the tumour, preferably in proportion to the volumes of the various parts, as discussed previously.

In view of these findings, it is legitimate to ask whether any useful information is being obtained using the method within the constraints of human experimentation. Results in non-tumorous tissues have been better but the ethical considerations relating to such investigations must not be ignored. Our first illustration (Fig. 1) shows mitotic index data obtained from the jejunum of a patient with morphologically normal mucosa, using serial biopsies with the Quinton hydraulic multiple biopsy tube *in situ*, after vincristine in a dose of 0·045 mg/kg, injected intravenously. The data have already been corrected (Tannock, 1967) by multiplication by 0·71 to compensate for preferential observation of mitoses. There was apparently no delay period nor evidence of anaphase escape and linearity may be assumed over the whole experimental period. The data were fitted assuming a rectangular age distribution and gives a birth rate of 25 cells/1000 cells per hr (95% confidence limits 20·4 and 29·4 cells/1000 cells per hr). This can be compared with the 30·1 and 36·4 cells/1000 cells per hr reported for two patients with normal jejunal mucosae, obtained with the FLM technique by Weinstein *et al.* (1973).

Similarly, in the common skin disease psoriasis, there is known to be an increase in epidermal proliferative rates (Weinstein & Frost, 1968). Duffill *et al.* (1977) present a metaphase accumulation curve derived from eight patients with stable psoriatic plaques, after intradermal injection of 1 μg of vincristine, followed by serial biopsies (Duffill, Wright & Shuster, 1976). The line was fitted assuming a growth fraction of unity and an exponential

age distribution (Appleton *et al.*, 1977). This gave a birth rate of 12·4 cells/1000 cells per hr with 95% confidence limits of 7·1 and 18·7. Results from an FLM curve on a patient with psoriasis gave a mean birth rate of 12·3 cells/1000 cells per hr, while calculation of the birth rate from the flash labelling index and t_s gave a value of 12·7 cells/1000 cells per hr (using the same assumptions as before). Such agreement is unusually good. It is not entirely clear whether it is better to find the mean of individual k_B estimates or to pool the data and find a single estimate, but the first method is normally to be preferred if there are enough observations for each subject.

Camplejohn *et al.* (1973) reported a value of 12·2 cells /1000 cells per hr for the birth rate in human rectal mucosa, as measured with vincristine; using the growth fraction of 0·5 and the cell cycle time of 42 hr derived from [^{3}H]TdR FLM studies by Lipkin, Sherlock & Bell (1963) we obtain 11·9 cells/1000 cells per hr. Furthermore, in human pyloric mucosa, Wright *et al.* (1977) reported a mean cell production rate of 13 cells/1000 cells per hr, again with vincristine, which also agrees reasonably with the 10 cells/1000 cells per hr found by Shorter *et al.* (1964) and given by Lipkin (1965).

It thus appears that, in studies of normal human tissues, values obtained with vincristine can be close to those given by the sophisticated [^{3}H]TdR techniques; this may be a consequence of the larger number of biopsies which are available in non-tumour tissues. We should, however, be guarded in our acceptance of such results unless optimal conditions for the stathmokinetic experiment are realized. It is also worth remembering that these comparisons are made on different individuals who may be in different metabolic states, and as in the experimental studies, we should really compare the two techniques carried out on the same individuals at the same time—probably an impossible objective.

Conclusions

It is clear from the numerous instances which have been quoted in this review that the metaphase arrest technique has been subject to a good deal of ill-treatment in recent years; such abuse led Clarke (1971) to observe that the method 'enjoys a certain disrepute to this day'. It has been our purpose to highlight the point that much of this disrepute stems from the ill-conceived manner in which many workers have applied the technique, and particularly in the several short-cuts which have been employed.

The metaphase arrest technique gives an estimate of a useful kinetic parameter, the cell birth rate; when data are analysed correctly an appreciation of their worth can be obtained from a confidence interval for the estimate, and if the age distribution is known we can calculate the turnover time or potential doubling time with confidence limits. It is our opinion that a confidence interval for the cell birth rate should always be quoted, as there are certain situations, especially in human work, where the wide confidence intervals are often more relevant than a single estimate for the birth rate. Indeed, if this method is to be used at all in the future, its limitations must be appreciated adequately.

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Virchows Arch. B Cell Path. 32, 69–73 (1979)

Virchows Archiv B
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8

Estimating the Proportion of Proliferating Cells in a Population

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Summary. In a population of cells that proportion which is actively engaged in the proliferative cycle is often estimated from the ratio of the observed labelling index to an expected labelling index, calculated, on the basis of all cells being in cycle, from the cell cycle phase durations and the age distribution.

Ignoring the variability in cell cycle times may lead to large overestimates or underestimates in the expected labelling index. A method is given of obtaining a more accurate estimate of this variable, and hence of the proliferative proportion.

Key words: Growth fraction – Variability.

Introduction

The proliferative proportion, or growth fraction, of a cell population may be found from the ratio of the observed proportion of cells in DNA-synthesis, taken to be the tritiated thymidine labelling index (I_s^{obs}), to that expected if all cells were cycling (I_s^{exp}). To calculate I_s^{exp} it is necessary to know the age distribution of cells and the cell cycle phase durations, which we shall denote by t_{G_1}, t_s, t_{G_2}, and t_m; the cell cycle time is T_c. For a rectangular age distribution Cleaver (1967) gives the equation

$$I_s^{exp} = t_s/T_c \tag{1}$$

and for an exponential age distribution his formula leads to

$$I_s^{exp} = 2^{1-t_{G_1}/T_c}\{1-2^{-t_s/T_c}\}. \tag{2}$$

These formulae assume that the variability in phase durations is negligible. Appleton, Wright and Dyson (1977) showed in another context (the analysis of

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0340-6075/79/0032/0069/$01.00

stathmokinetic experiments) that this assumption could lead to large errors. It is thus advisable to examine the effect of ignoring variability in autoradiographic experiments conducted for the purpose of finding the growth fraction, and we shall show that once again large errors may be made and that the estimates obtained from Eqs. (1) and (2) may be either overestimates or underestimates depending on the phase durations and their variability. A table of approximate correction factors, and a short computer program which obtains better estimates will be given.

The Distribution of Times to Mitosis

If the age distribution $y(x)$ satisfies the equation of Steel (1968)

$$y(x)=\frac{1+p}{p}\frac{\log(1+p)}{T_c}\exp\left\{-\frac{\log(1+p)}{T_c}x\right\} \tag{3}$$

then $p=1$ gives an exponential age distribution and the limit as $p\to 0$ gives a rectangular age distribution. Appleton et al. (1977) showed that, if T_c is itself subject to variation and follows a gamma distribution with mean n/λ and coefficient of variation (CV) $n^{-\frac{1}{2}}$, the distribution $g(x)$ of times remaining to mitosis is given by

$$g(x)=\frac{1+p}{p}\log(1+p)\frac{\lambda^n}{\Gamma(n)}\int_0^\infty (t+x)^{n-2}\cdot\exp\left\{\frac{-t\log(1+p)}{t+x}\right\}\cdot\exp\{-\lambda(t+x)\}\,dt. \tag{4}$$

For the rectangular age distribution this may conveniently be written in terms of a mean T_c of one unit as

$$g(x)=\frac{n e^{-nx}}{\Gamma(n)}\int_0^\infty (t+nx)^{n-2}e^{-t}\,dt \tag{5}$$

and, since the method is almost identical for other values of p, we shall present only this common situation, though the program in the appendix treats the general case. The equation is of the form

$$g(x)=a(x)\int_0^\infty b(x,t)\,e^{-t}\,dt$$

and hence may be conveniently evaluated by Gauss-Laguerre quadrature (Stroud and Secrest, 1966); the 16 point formula with points and weights correct to 4 figures provides sufficient precision. The expected labelling index

$$I_s^{\exp}=\int_{(t_{G_2}+t_m)/T_c}^{(t_s+t_{G_2}+t_m)/T_c} g(x)\,dx \tag{6}$$

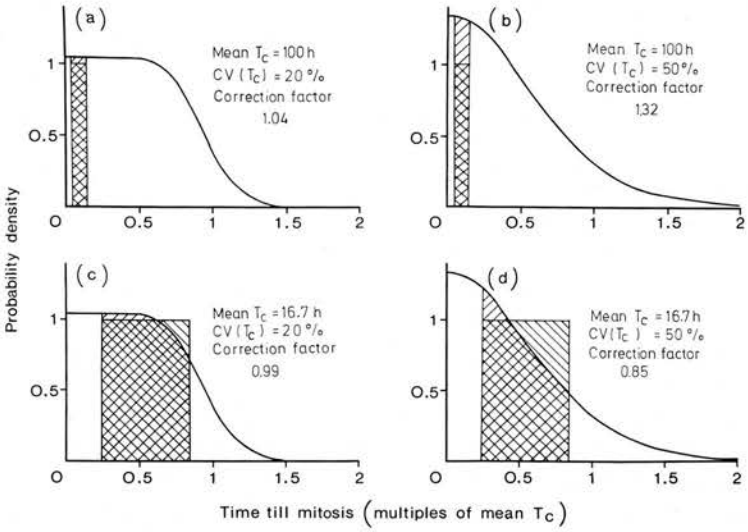

Fig. 1a–d. Distribution of time till mitosis for a basically rectangular age distribution, with variability only in t_{G_1}. All figures have $t_s = 10$ h, $t_{G_2} + t_m = 4$ h. (**a**) and (**b**) have $t_{G_1} = 86$ h; (**c**) and (**d**) 2.7 h. (**a**) and (**c**) have $CV(T_c) = 20\%$, (**b**) and (**d**) 50%. ▨ represents labelled cells, ▩ represents cells taken to be labelled if the growth fraction is calculated as t_s/T_c

may then be calculated by using Simpson's rule; it is not necessary to evaluate $g(x)$ for more than 3 values of x to obtain satisfactory precision. Equations (5) and (6) lead to

$$I_s^{\exp} \simeq \frac{n}{6 t_s \Gamma(n)} \{S(t_m + t_{G_2}) + 4S(t_m + t_{G_2} + \tfrac{1}{2} t_s) + S(t_m + t_{G_2} + t_s)\} \tag{7}$$

where

$$S(\tau) = e^{-n\tau/T_c} \sum_{i=1}^{16} A_i (t_i + n\tau/T_c)^{n-2}$$

and A_i and t_i are the i^{th} 16 point Gauss-Laguerre weight and point.

This method of defining $I_s^{\exp}$ assumes that t_s, t_{G_2} and t_m may be considered constant and that the variability in T_c consists entirely of variability in t_{G_1}; an assumption inconsistent with a gamma distribution for T_c, but not with the general shape of the distribution. We shall present our results in terms of the coefficient of variation of the distribution.

Numerical Example

Consider the case where $t_m = 1$ h, $t_{G_2} = 3$ h and $t_s = 10$ h. We can calculate that the values of T_c required to give uncorrected $I_s^{\exp}$ values of 10, 20, 30, 40, 50 and 60 % are 100, 50, 33.3, 25, 20 and 16.7 h respectively for a rectangular age distribution; for CVs of 20, 30, 40, 50, 60, 70 and 80 % we use values of n equal to 25, 11.11, 6.25, 4, 2.78, 2.04 and 1.56 respectively.

Table 1. Approximate correction factors by which to multiply an estimate of the expected labelling index, I_s^{exp}, calculated as t_s/T_c (rectangular age distribution) when the coefficient of variation of the cell cycle time, T_c, is known

		Coefficient of variation of T_c (%)						
		20	30	40	50	60	70	80
Initial estimate of I_s^{exp}	10	1.04	1.10	1.19	1.32	1.49	1.66	1.80
	20	1.04	1.10	1.18	1.28	1.36	1.40	1.42
	30	1.04	1.09	1.15	1.19	1.20	1.19	1.15
	40	1.04	1.08	1.09	1.08	1.05	1.00	0.95
	50	1.03	1.03	1.01	0.97	0.91	0.85	0.79
	60	0.99	0.95	0.91	0.85	0.79	0.72	0.67

Figure 1 illustrates 4 particular cases, and Table 1 shows I_s^{exp} as evaluated from Eq. (7) divided by the corresponding value from Eq. (1). These tabulated values may therefore be used to multiply uncorrected values of I_s^{exp} to obtain corrected values. It would be possible similarly to construct a table of correction factors for the case of the exponential age distribution, but we would prefer workers to calculate their estimates of I_s^{exp} directly, and for this purpose a computer program is given in the appendix.

Discussion

The correction factors in Table 1 vary from less than 0.7 to 1.8. It is clear therefore that large errors may be incurred by ignoring the variation in cell cycle time when using the ratio of observed to expected labelling index to estimate the growth fraction; and that these errors may be such as to overestimate or underestimate that quantity. Greatest errors are made when the cell cycle is very long or very short (so that the S-phase is close to one end) and T_c is very variable. Table 1 is intended to demonstrate the magnitude of the problem; it should not be used to find correction factors for initial estimates of I_s^{exp}, having been produced for a specific age distribution and specific phase durations.

Even the results of the full version of the method, as portrayed in the computer program, should not be used indiscriminately. The six items of data input are all approximations, and so must the answer be. In addition the method is derived from a less detailed model than that, for instance, of Steel (1977). However, we feel that its basic simplicity means that all workers will be able to understand it and use it routinely, which might not be so with a more rigorous approach.

Appendix: An APL Computer Program Which Calculates I_s^{exp}

The program given below should work on any computer supporting APL. It takes as data p (see Eq. (3) in the text), the CV of T_c (as a percentage), t_{G_1}, t_s, t_{G_2},

and t_m; it returns a value for I_s^{exp} by the method described in the text. If the CV of T_c is less than 20% interpolation is used between the value of I_s^{exp} for a CV of 20% and the value found by ignoring variability. Examples of the use of the program are given for an exponential age distribution with no variability, an exponential distribution with a CV of 50% in T_c, and a rectangular distribution, again with a CV of 50% in T_c.

```
    ∇Z←A ISEXP T;N;P;X;V;GLX;GLW
[1] →(∨/(2≠ρA),(4≠ρT),(0>T,A),A>1 100)/E
[2] GLX←0.08765 0.4627 1.141 2.129 3.437 5.078 7.070 9.438
    12.21 15.44 19.18 23.52 28.59 34.58 41.94 51.70
[3] GLW←*−1.579 1.105 1.325 1.993 3.051 4.483 6.293 8.496
    11.12 14.20 17.79 21.97 26.88 32.70 39.83 49.23
[4] X←(+/(3 4ρ0 0 1 1 0 0.5 1 1 0 1 1 1)×3 4ρT)÷+/T
[5] →(A[2]<20)/L,P←1↑A,N←25
[6] P←1↑A,N←10000÷A[2]*2
[7] L:Z←++/(⍉3 16ρGLW)×((GLX∘.+N×X)*N−2)×(1+P)*−÷1
    +(÷GLX)∘.×N×X
[8] Z←(((1+P)×((⊛1+P)÷P)×N÷!N−1)×*−N×X)×Z
[9] →(A[2]≧20)/0,Z←+/(1 4 1×T[2]÷6×+/T)×Z
[10] V←(((1+P)*1−T[1]÷+/T)×1−(1+P)*−T[2]÷+/T)+(P=0)
    ×T[2]÷+/T
[11] →0,Z←V+(Z−V)×(0.05×A[2])*2
[12] E:'ERROR IN DATA'
    ∇

    1 0 ISEXP 26 10 3 1
0.203

    1 50 ISEXP 26 10 3 1
0.278

    0 50 ISEXP 26 10 3 1
0.310
```

Acknowledgements. We are grateful to Dr. A.J. Watson for his helpful comments and to Mrs. P. Thompson for typing the manuscript.

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Received July 23, 1979

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The highest labelled cell in the intestinal crypt column

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Running title: The highest labelled cell

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Abstract

A formula is given, and proved, for the expected position of the highest labelled cell in an individual crypt column, if the labelling index distribution is known.

Introduction

Among the several possible methods of assessing the spatial extent of proliferative activity in the intestinal crypt are the determination of the cut-off position (Cairnie, Lamerton and Steel, 1965), estimation of the half-maximum position of the labelling index distribution (Cleaver, 1967 p211), and calculation of the mean position of the highest labelled cell, over a number of crypt columns (Hamilton, 1979). Wright and Alison (1984 p617) attribute to us a formula for estimating the last of these from the labelling index distribution. In this article we give a proof of the formula, and from it demonstrate a problem in the use of the mean position of the highest labelled cell as a statistic to describe the extent of the proliferative compartment.

Theorem

If the cell at cell position j from the bottom of a column k cells high has probability p_j of being labelled, then the expected (mean) position λ of the highest labelled cell, given that at least one is labelled, is

$$\lambda = \frac{k - \sum_{i=1}^{k} \prod_{j=i}^{k} (1-p_j)}{1 - \prod_{j=1}^{k} (1-p_j)}.$$

Note that the formula is misprinted in Wright and Alison (1984).

Proof

The probability that cell i is labelled, but cells i+1, i+2, ..., k are not is

$$p_i \prod_{j=i+1}^{k} (1-p_j).$$

By definition, therefore, the expected value of i is

$$\frac{\sum_{i=1}^{k} i\, p_i \prod_{j=i+1}^{k} (1-p_j)}{\sum_{i=1}^{k} p_i \prod_{j=i+1}^{k} (1-p_j)} .$$

Now,

$$\sum_{i=1}^{k} p_i \prod_{j=i+1}^{k} (1-p_j) = \sum_{i=1}^{k} \prod_{j=i+1}^{k} (1-p_j) - \sum_{i=1}^{k} \prod_{j=i}^{k} (1-p_j)$$

$$= 1 + \sum_{i=2}^{k} \prod_{j=i}^{k} (1-p_j) - \sum_{i=1}^{k} \prod_{j=i}^{k} (1-p_j)$$

$$= 1 - \prod_{j=1}^{k} (1-p_j)$$

and,

$$\sum_{i=1}^{k} i\, p_i \prod_{j=i+1}^{k} (1-p_j) = \sum_{i=1}^{k} i \prod_{j=i+1}^{k} (1-p_j) - \sum_{i=1}^{k} i \prod_{j=i}^{k} (1-p_j) .$$

$$= k + \sum_{i=1}^{k-1} i \prod_{j=i+1}^{k} (1-p_j) - \sum_{i=1}^{k} i \prod_{j=i}^{k} (1-p_j)$$

$$= k + \sum_{i=2}^{k} (i-1) \prod_{j=i}^{k} (1-p_j) - \sum_{i=1}^{k} i \prod_{j=i}^{k} (1-p_j)$$

$$= k - \prod_{j=1}^{k} (1-p_j) - \sum_{i=2}^{k} \prod_{j=i}^{k} (1-p_j)$$

$$= k - \sum_{i=1}^{k} \prod_{j=i}^{k} (1-p_j)$$

These two relationships prove the required result.

Application

For the labelling index distribution of figure 1, which is a composite curve for 100 caecal crypts in each of 4 rats (Sunter et al, 1979), not expressed to a standard crypt, the theoretical value of λ is 10.9, which agrees well with the actual value of 10.3 calculated directly from the 385 crypts with labelled cells.

The problem with this measure can be demonstrated by evaluating λ for other sets of values of p_j, where the new values are constant multiples of those observed. Table 1 shows that λ increases with the labelling index, although the proliferative compartment would, according to the half-maximum position, not be considered to have changed. Indeed, if indices one-tenth of those observed were used (as they might be when dealing with the mitotic index) λ would be three cells lower. Of course, if the mean position of the highest labelled cell changes greatly while there are only small changes in labelling index, as in the data of Hamilton (1979), there is no ambiguity of interpretation.

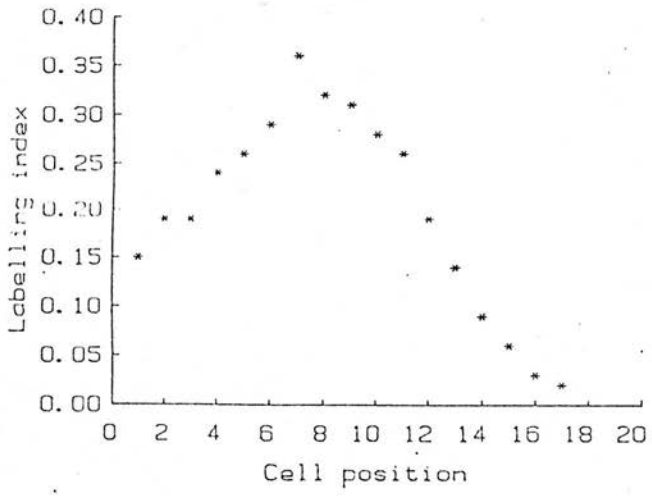

Figure 1. Labelling index at each cell position in the rat caecum, counting from the crypt base. Results are averaged over 100 crypt columns in each of 4 animals.

Table 1

Each of the labelling indices in figure 1 has been multiplied by the 'multiplier'. λ is the expected position of the highest labelled cell which results.

Multiplier	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
λ	8.5	9.4	10.2	10.9	11.5	11.9	12.3	12.6

Computer program

It is possible to evaluate the expression for λ using a short APL computer program, e.g.

```
∇ Z←TOPPOINT P;K;N
[1] Z←N<⌽N←(K,K)⍴1↓K←⍴P←1-P
[2] Z←(K-+/×/Z⌈(⍴N)⍴P)÷1-×/P
∇
```

P represents the labelling indices p_1, p_2, ... p_k, so the call of the program

```
TOPPOINT 15 19 19 24 26 29 36 32 31 28 26 19 14 9 6 3 2 0 0 0 ÷ 100
```

returns the result 10.9 as given above.

Discussion

There is no doubt that it is easier to calculate the mean position of the highest labelled cell than to produce the entire labelling index distribution, but if the former method is used information is lost, and it may be difficult to separate a change in the labelling index brought about by an altered cell cycle time from a change in the extent of the proliferative compartment. Other methods also suffer from this difficulty to some extent, and it is probable that only simulations of the crypt can adequately describe its kinetics.

Several other tissues share with the intestinal crypt the property that cells migrate in one direction, and they may be looked at in a very similar way in terms of having a highest labelled cell or a labelling index distribution. Such tissues include the root meristem (Barlow, 1973), the cartilage growth plate in long bones (Kember, 1978), and the rat incisor (Zajicek, Michaell and Regev, 1979).

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10

The Age Distribution of Cells in Stratified Squamous Epithelium

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(*Received* 27 *May* 1976, *and in revised form* 7 *October* 1976)

In structured tissues such as stratified squamous epithelium the age distribution of cycling cells in a particular part of the tissue is influenced by cell migration. Age distributions are derived for the proliferative cells of stratified squamous epithelium, dependent on the mode of migration; and the effect of using analytical methods which assume exponential or rectangular age distributions is discussed with particular reference to the analysis of stathmokinetic experiments.

1. Introduction

Many methods of estimating the cell kinetic parameters of a tissue make assumptions about the age distribution of cells which are satisfied at best only approximately. It is frequently assumed that the age distribution is that which would arise in a population of cells growing exponentially with a constant cell cycle time, but little is known about the magnitude of the errors which this simplification entails. It is the purpose of this article to consider how estimates of the mean cell cycle time derived from stathmokinetic experiments in stratified squamous epithelium will be affected by cell migration in the tissue, and to derive better methods of estimating the cell cycle time from knowledge of the proportion of mitoses whose axes are in the plane of the basement membrane or perpendicular to it. The case of the single basal proliferative layer found in most squamous epithelium will be considered as a particular example of the general case of any number of layers, the latter being relevant, for example, to hyperplastic epithelium or psoriatic epidermis.

2. The Mathematical Model

Consider a layered structure of proliferative cells in which all cells have the same cell cycle time T. (This restriction will be removed later.) Suppose that there are several layers of cells, and that for any layer k at each mitosis

one daughter cell at least remains in that layer. With probability p_k the other cell also remains, otherwise it moves to layer $k+1$ displacing a cell from that layer into layer $k+2$; such a displaced cell is chosen at random with respect to its age. If both daughter cells remain in layer k a cell from that layer is displaced into the next layer, and so on, each cell displaced in turn displacing a cell in the layer above. A value for p_k may be obtained from observation of the proportion of mitoses in layer k which have their axis (i.e. the line joining the nuclei of the two daughter cells) in the plane of the layer.

The age distribution of a cell population is a function which allows us to calculate the probability that a cell chosen at random is of a particular age; it tells us what proportion of cells we may expect to be of a given age. If the age distribution of cells in layer k is $y_k(x)$ then, with the convention that $y_0(x) = 0$, and except for very young cells and those near the end of mitosis which are dealt with in equation (2) we have,

the proportion of cells in layer k of age $x+\mathrm{d}x$

$$y_k(x+\mathrm{d}x)$$

is the same as the proportion of age x

$$= y_k(x)$$

less those cells of that age which have been pushed out by the horizontal mitoses in that layer during time $\mathrm{d}x$,

$$-y_k(x)y_k(T)p_k\,\mathrm{d}x$$

less those pushed out by any mitosis in any of the lower $k-1$ layers,

$$-\sum_{i=1}^{k-1} y_k(x)y_i(T)\,\mathrm{d}x$$

plus those pushed up from the layer below by horizontal mitoses in that layer,

$$+y_{k-1}(x)y_{k-1}(T)\;p_{k-1}\;\mathrm{d}x$$

plus those pushed up by any mitosis in the $k-2$ layers below that.

$$+\sum_{i=1}^{k-2} y_{k-1}(x)y_i(T)\,\mathrm{d}x.$$

If we write

$$C_k = p_k y_k(T) + \sum_{i=1}^{k-1} y_i(T)$$

then this equation may be expressed in the form of a differential equation.

$$y_k'(x)+C_k y_k(x) = C_{k-1} y_{k-1}(x)$$

which has solution

$$y_k(x) = A_k \, e^{-C_k x}+C_{k-1} \, e^{-C_k x} \int_0^x y_{k-1}(x) \, e^{C_k x} \, dx \tag{1}$$

where A_k is a constant chosen to satisfy the boundary conditions, which may be expressed either as

$$\int_0^T y_k(x) \, dx = 1$$

or

$$y_k(0) = (1+p_k)y_k(T)+(1-p_{k-1})y_{k-1}(T). \tag{2}$$

In a stathmokinetic experiment cells are arrested in mitosis by an agent such as vincristine and the mitotic index calculated at intervals thereafter. If $M_k(t)$ is the mitotic index in layer k after time t during such an experiment, then

$$M_k(t) = \int_{T-t_m-t}^{T} y_k(x) \, dx$$

where t_m is the mitotic duration. Using equation (1),

$$M_k(t) = A_k \int_{T-t_m-t}^{T} e^{-C_k x} \, dx+C_{k-1} \int_{T-t_m-t}^{T} e^{-C_k x} \, dx \int_0^x y_{k-1}(\xi) \, e^{C_k \xi} \, d\xi.$$

Changing the order of integration of the double integral gives

$$M_k(t) = \frac{1}{C_k} \{y_k(T-t_m-t)-y_k(T)+C_{k-1} M_{k-1}(t)\}. \tag{3}$$

An approximation which will prove useful is

$$M_k(t) \simeq (t_m+t)y_k(T) \tag{4}$$

and in particular the native mitotic index is given by

$$M_k(0) \simeq t_m y_k(T).$$

3. The Basal Layer

For the first (or only) layer of cells equation (1) reduces to

$$y(x) = A \, e^{-cx}$$

where $c = py(T)$. Equation (2) becomes

$$y(0) = (1+p)y(T),$$

and the solution may be written in the form

$$y(x) = \frac{1+p}{p} \frac{\log(1+p)}{T} \exp\left\{\frac{\log(1+p)}{T} x\right\}. \tag{5}$$

The formal similarity of this equation to that of Steel (1968) should be noted; the function is illustrated for different values of p in Fig. 1.

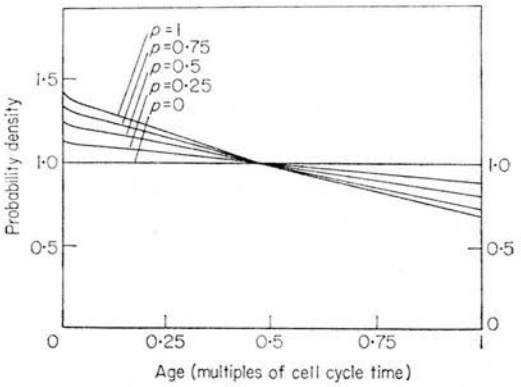

FIG. 1. The age distribution $\frac{1+p}{p} \frac{\log(1+p)}{T} \exp\left\{-\frac{\log(1+p)}{T} x\right\}$ of cells in the basal layer for values of p, the proportion of mitotic axes in the plane of the layer, 0, 0·25, 0·5, 0·75 and 1.

Equation (3) reduces to

$$M(t) = \frac{1}{c}\{y(T-t_m-t) - y(T)\}$$

whence,

$$\log_{1+p}\{1+pM(t)\} = \frac{1}{T}(t_m+t). \tag{6}$$

In the limit as $p \to 0$ this becomes

$$M(t) = \frac{1}{T}(t_m+t), \tag{7}$$

while for $p = 1$ we have Puck & Steffen's (1963) "collection function" which is usually written as

$$\log_{10}\{1+M(t)\} = \frac{\log_{10} 2}{T}(t_m+t).$$

The native mitotic index is given by

$$M(0) = \frac{t_m}{T} \frac{\log (1+p)}{p}.$$

4. Numerical Results

Although it is best to estimate the cell cycle time from experimental data by fitting equation (6), and an example of this will be given later, it is informative to look more closely at equation (4). This approximation is valid if t_m+t is small compared to T, and since a stathmokinetic experiment is usually short compared to the cell cycle time the approximation is normally a good one. For the basal layer we therefore have, using equation (5)

$$M(t) \simeq \frac{\log (1+p)}{p} \frac{1}{T} (t_m+t).$$

The values of $[\log (1+p)]/p$ for $p = 0$, 0·25, 0·5, 0·75 and 1 are 1·00, 0·89, 0·81, 0·75 and 0·69 respectively; these may be thought of as correction factors by which to multiply the estimate of the cell cycle time found by fitting equation (7).

5. Higher Layers

One extreme case is that in which $p_k = 1$ for all k, i.e. the mitotic axis is always in the plane of the basal layer. We may show that for all k

$$y_k(x) = 2 \frac{\log 2}{T} \exp \left\{ - \frac{\log 2}{T} x \right\}$$

and

$$M_k(t) = \exp \left\{ \frac{\log 2}{T} (t_m+t) \right\} - 1,$$

whence

$$\log_{10} \{1+M(t)\} = \frac{\log_{10} 2}{T} (t_m+t).$$

In other words, the age distribution of each layer and hence for the tissue as a whole is that found under conditions of exponential growth.

At the other extreme (in a sense) is the case for which $p_k = 0$ for all k, i.e. all mitotic axes are perpendicular to the layers of cells. The limiting value of equation (5) gives

$$y_1(x) = \frac{1}{T}.$$

Using this in equation (1) it can be shown that

$$y_2(x) = \frac{\kappa_2}{T} e^{-x/T}$$

where

$$\kappa_2 = e/(e-1) \simeq 1{\cdot}582.$$

For the third layer

$$y_3(x) = \frac{\kappa_3}{T} e^{-\kappa_2 x/T} + \frac{1}{T} e^{1-x/T}$$

where

$$\kappa_3 = \kappa_2(2-e)/(1-e^{-\kappa_2}) \simeq -1{\cdot}430.$$

These age distributions are shown in Fig. 2.

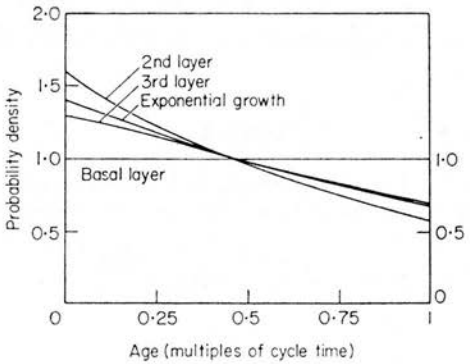

FIG. 2. Age distributions of cells in the first three layers of stratified squamous epithelium when all mitotic axes are perpendicular to the plane of the basal layer.

The corresponding expressions for $M_k(t)$ are

$$M_1(t) = \frac{1}{T}(t_m + t)$$

$$M_2(t) = \frac{1}{e-1}\left\{\exp\left(\frac{t_m+t}{T}\right) - 1\right\}$$

$$M_3(t) = \left\{\exp\left(\frac{t_m+t}{T}\right) - 1\right\} + \frac{\kappa_3}{\kappa_2} e^{-\kappa_2}\left\{\exp\left\{\frac{\kappa_2(t_m+t)}{T}\right\} - 1\right\}.$$

6. Numerical Results

Again using equation (4) and ignoring terms of higher order than $(t_m+t)/T$ we can write $M_k(t) = (\gamma_k/T)(t_m+t)$; values of γ_k for $k = 1, 2$ and 3 are 1·00, 0·58 and 0·71. The age distribution for higher layers becomes progressively nearer that for exponential growth conditions (i.e. $\gamma_k \to 0{\cdot}69$ as $k \to \infty$). It should be noted, however, that the values of γ_k are not all between 1 and 0·69. If layer k has a high value for γ_k it provides layer $k+1$ with a high proportion of young cells and so γ_{k+1} will be low.

It can be seen from the variation in these values that we commit considerable errors in analysing experimental data for any layer above the basal layer assuming a rectangular age distribution, even if that is accurate for the basal layer. We also must expect to obtain erroneous estimates for the cell cycle time if we analyse data from the second layer using any expression other than the one derived explicitly for it.

The variation in the γ_k for different k also indicates that the native mitotic index is a very misleading index of proliferation, for in the situation just described the second layer of cells will contain fewer than 60% as many mitoses as the basal layer in spite of the fact that all the cells in both layers are in cycle. The age distribution which obtains in the second layer is not indeed the most extreme possible: if mitotic axes in the basal layer were perpendicular to the layer ($p_1 = 0$) while those in the second layer were in the plane of the layer ($p_2 = 1$), $M_2(0)$ would be approximately $0{\cdot}45(t_m/T)$.

It is fortunate, however, that the age distribution for a many-layered structure of proliferating cells *taken as a whole* is sufficiently close to the exponential age distribution, irrespective of the p_k, for methods based on this assumption to be accurate enough for most practical purposes.

The age distributions for the first three layers with $p_1 = p_2 = p_3 = 0$, and that for exponential conditions, are shown in Fig. 1.

7. Variation in the Cell Cycle Time

The model so far considered has assumed a constant cell cycle time; we now extend it to allow the cell cycle time to vary according to a known distribution. The family of distributions used should not affect results to any great extent except when variability is very high [compare Hartmann *et al.* (1975), and we choose to assume for simplicity that the cell cycle time distribution $q(T)$ is a gamma distribution with mean n/λ and coefficient of variation $n^{-\frac{1}{2}}$.

i.e.,
$$q(T) = \frac{1}{\Gamma(n)} \lambda^n T^{n-1} \mathrm{e}^{-\lambda T}. \tag{8}$$

If $y(x|T)$ is the age distribution given that the cell cycle time is T, then the overall age distribution is

$$f(x) = \int_x^\infty y(x|T)q(T)\,\mathrm{d}T.$$

More useful for our purposes is the distribution of times remaining to mitosis, $g(x)$.

$$g(x) = \int_x^\infty y(T-x|T)q(T)\,\mathrm{d}T = \int_0^\infty y(t|t+x)q(t+x)\,\mathrm{d}t.$$

Considering the basal layer, and writing $\theta = \log(1+p)$, we have from equations (5) and (8),

$$g(x) = \frac{1+p}{p}\,\theta\,\frac{\lambda^n}{\Gamma(n)}\int_0^\infty (t+x)^{n-2}\exp\left\{\frac{-\theta t}{t+x}\right\}\exp\{-\lambda(t+x)\}\,\mathrm{d}t.$$

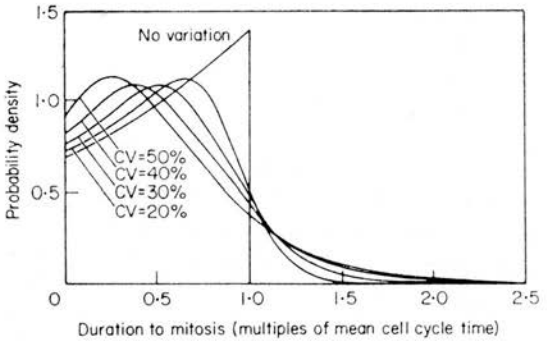

FIG. 3. Distribution of duration to mitosis in the basal layer when all mitotic axes are in the plane of the layer for values of the coefficient of variation of cell cycle time 20, 30, 40 and 50%; a gamma distribution is assumed for cell cycle time.

Figure 3 illustrates $g(x)$ for the case $p = 1$ and various values of the coefficient of variation of the distribution of cell cycle times; these density functions were evaluated using the 16-point Gauss–Laguerre integration formula (Stroud & Secrest, 1966). For larger, but by no means unheard of, coefficients of variation the distributions differ quite markedly from that obtained by assuming no variation. These differences are reflected in their effect on the analysis of stathmokinetic experiments. The rate of entry into mitosis at the start of the experiment is

$$g(0) \simeq \frac{n}{n-1}\,\frac{\log(1+p)}{p}.$$

The values of the ratio $n/(n-1)$ corresponding to coefficients of variation of 20, 30, 40 and 50% are 1·04, 1·10, 1·19 and 1·33 respectively. These ratios may again be thought of as correction factors; the estimate of cell cycle time obtained from the simpler model should be multiplied by the appropriate factor to obtain a better estimate of the mean cell cycle time.

Unfortunately estimates of the required coefficient of variation are seldom available, and often little can be said except that there is considerable variation and the estimate of the cell cycle time obtained is likely to be an underestimate by an indeterminate amount. Sometimes it is possible to quantify the variability, and Fig. 4 is intended as a guide to appreciating

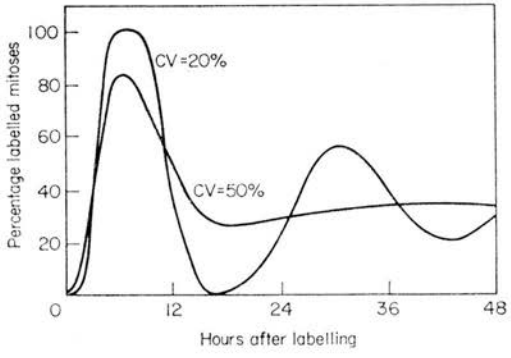

FIG. 4. FLM curves for exponential growth conditions with mean phase times: G_1 12 h, S 8 h, G_2 3 h, M 1 h. The coefficient of variation for the cell cycle time (20 and 50%) arises from equal CVs for S, G_2 and M, and the CV for G_1 twice that value; individual phase times are gamma distributed.

what the coefficient of variation represents. It is the output from an adaptation of the Gilbert (1972) FLM program and shows FLM curves for mean phases of the cell cycle as indicated and coefficients of variation of the cell cycle time of 20 and 50%; there are many such curves possible and the ones shown have coefficients of variation for G_1 twice that for the other phases of the cycle. A coefficient of variation of 50% is more than might be expected in most experimental tissues (but not tumours) while 20% would probably be considered agreeably low.

8. Estimating the Cell Cycle Time from Experimental Data

Twenty-two male A_2G mice aged between 7 and 8 months (which had been treated with urethane 14 weeks previously) were used in a stathmokinetic experiment to determine the cell cycle time of cells in the basal

layer of the oesophagus. The proportion of mitoses with axes in the plane of the layer was assessed from five mice, and the other 17 were given 1 mg per kg body weight of vincristine sulphate (Oncovin, Lilly) by intraperitoneal injection at 09.00 h; one mouse was killed by cervical dislocation every 15 min until 4·25 h after injection. The oesophagi were fixed in Carnoy's fluid for 6 h and embedded in paraffin. Transverse sections were cut at a thickness of 5 μm and were stained with Harris's haematoxylin.

Apart from the reading at 2 h after vincristine for which less tissue was available the mitotic index was calculated on the basis of at least 2000 cells. Telophases were observed in the sections from animals killed up to 75 min,

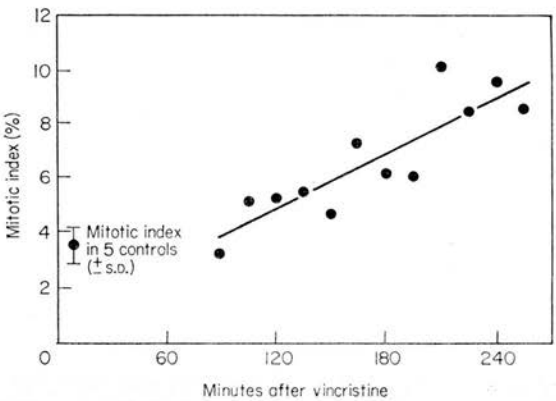

FIG. 5. Mitotic index [$M(t)$] in basal layer of mouse oesophagus as a function of time (t) after vincristine; the fitted curve is of the form $\log_{1+p}\{1 + pM(t)\} = (1/T)(t_m + t)$ with $p = 0{\cdot}37$ and gives an estimate of 43 h for T.

and these observations have been excluded from the calculations of cell cycle time, since the vincristine could not have taken full effect. Figure 5 shows the mitotic index as a function of time after vincristine in the 12 animals killed from 90 min onwards.

Fifty-eight of the 155 mitoses whose direction of mitotic axis was determined were in the plane of the basal layer; 82 axes were clearly perpendicular to the layer and 15 were intermediate and have been treated as if they were perpendicular as one of the daughter cells probably left the basal layer. The best estimate of p, the proportion of mitotic axes in the plane of the layer, is therefore 0·37. The estimates of cell cycle time found from fitting equation (6) to the data with $p = 0$, 0·37 and 1 are 49, 43 and 36 h; Fig. 5 shows the fitted line corresponding to $p = 0{\cdot}37$. It should be stressed that the other lines ($p = 0$ and 1) appeared to fit the data equally well and it is

clearly not possible to use stathmokinetic data to determine the shape of the age distribution.

The value of 43 h obtained for the cell cycle time using $p = 0{\cdot}37$ has 95% confidence limits, based on the regression analysis, of 32 h and 67 h. Furthermore, these limits will be widened if account is taken of other uncertainties, both technical and theoretical, involved in the stathmokinetic technique. In view of this it may seem superfluous to go to the lengths we have to obtain such a poor estimate, but there is nevertheless a discrepancy of 6 or 7 h in the estimates given by the models which assume a rectangular or an exponential age distribution, and it is worth while trying to eliminate this error.

The fact remains that in spite of their apparent simplicity stathmokinetic experiments must not be expected to yield good approximations to the cell cycle time; other techniques are much more robust to departures from the assumptions on which their analysis is based.

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PSORIASIS: CELL PROLIFERATION

11

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CHURCHILL LIVINGSTONE
EDINBURGH LONDON MELBOURNE AND NEW YORK 1983

3. The fraction labelled mitoses method — general considerations

David R. Appleton

Since its conception by Howard and Pelc (1953) the fraction labelled mitoses (FLM) method has found widespread use in the study of cell proliferation, both in vitro and in vivo, of many plant, animal and human tissues. In some circumstances experimental results may easily be analysed and interpreted, giving precise and accurate estimates of the durations of the phases of the cell cycle and of the proportion of proliferating cells in the population studied. Sometimes, however, this is not so, and it is the purpose of this article to highlight the limitations of the method.

Briefly the experimental technique involves making available for a short time a radioactively labelled DNA precursor; we shall suppose this to be tritiated thymidine (^{3}HTdR) and assume for the present that it labels all cells which are in DNA synthesis when it is given, and no others. Thereafter, at short (e.g. hourly) intervals, mitoses are examined for the presence of label, and the number of labelled mitoses is expressed as a fraction of the total number of mitoses. In the ideal case when all cycling cells are moving asynchronously through the cell cycle with identical phase durations the graph of FLM against time will be as shown in Figure 3.1. Until the cells which were at the end of the S phase when label was introduced have passed through G_2 there will be no labelled mitoses; for a time equal to the duration of mitosis the fraction of mitoses labelled will increase linearly until it reaches unity, where it will remain until the cells which were at the beginning of the S phase at the time of labelling enter mitosis. Thereafter the labelled fraction falls to zero as these cells complete mitosis. When one cell cycle time has passed from the introduction of label the daughters of all cycling cells occupy the same position in the cycle as their parent cells and the process is repeated exactly.

The durations of the phases of the cell cycle (G_1, S, G_2 and M) can be estimated as shown in the figure. If label is available for an appreciable time, the duration of S will be overestimated and that of G_1 underestimated.

In practice counting error and variability between sections and animals in their cell proliferation characteristics makes estimation more difficult. Foremost among the causes of a departure from the simple diagram of Figure 3.1 is variability in cell cycle time which smooths the corners of the curve and lowers the

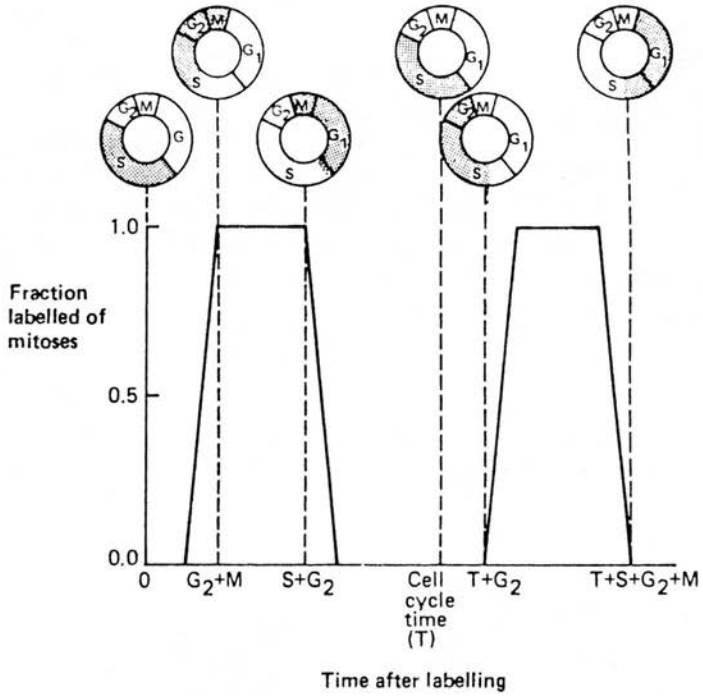

Fig. 3.1 A schematic representation of the cell cycle, showing the progress of labelled cells and the resulting FLM curve if all proliferating cells are cycling asynchronously with the same phase durations.

second, and possibly the first, peak. Figure 3.2 illustrates the sort of curves we can expect if the coefficient of variation (CV) of cell cycle times (the standard deviation as a percentage of the mean) is 20% or 50%.

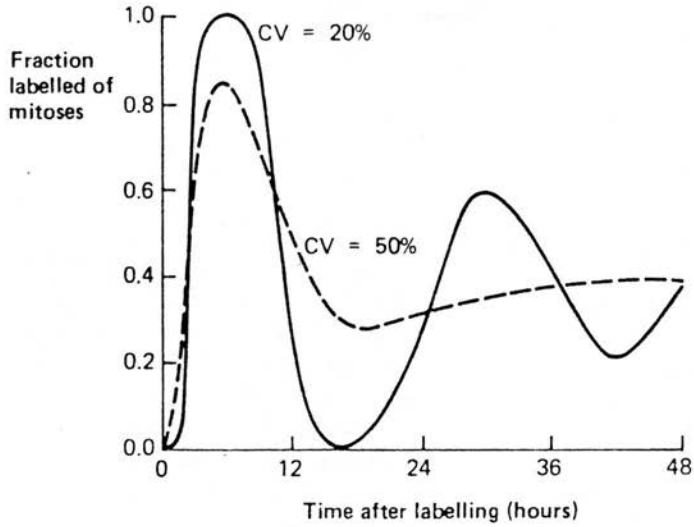

Fig. 3.2 FLM curves for conditions of exponential growth with gamma distributed phase times; G_1 12h, S 8h, G_2 3h and M 1h. The CV for the cell cycle time (20% and 50%) arises from equal CV's for S, G_2 and M and the CV for G_1 twice as high.

There are other difficulties with the method which we shall not consider here, as they are dealt with by others in this book in their discussions of other autoradiographic methods: failure of thymidine to reach the cells, failure of label to penetrate the emulsion, background counts, dilution of label with successive cell divisions, reutilisation of thymidine from dead cells, and the possible effect on the cell cycle kinetics of the very administration of ^{3}HTdR. Further details are also given by Steel (1977). Instead we shall concentrate on the effects of synchrony and variation between cells in their phase durations. We shall do this by means of simulation.

SIMULATIONS

Various methods are in use for the analysis of data from FLM experiments, and these have been compared (Hartmann et al, 1975) in relatively simple situations. Since they have been found to give reasonably similar results we shall concentrate on one: that due to Gilbert (1972). This method estimates the means and coefficients of variation for the distributions of times spent in G_2, $S + G_2$, and the entire cell cycle assuming that the phase durations are gamma distributed and that cells are cycling asynchronously. We shall investigate how it performs when its assumptions are violated, by simulating experimental data using the CELLSIM system of Donaghey (1975), which allows us to produce cell kinetic systems of virtually any degree of complexity. We shall first determine how extreme variability affects the analytical method, then describe the effect on analysis of synchrony caused by a cytotoxic insult, and we shall finally investigate a model where proliferation follows a more complex pattern than simple progress round the cell cycle. Although much can be done mathematically to investigate the properties of the FLM experiment, based on the methods of Barrett (1966) or MacDonald (1970), more complex systems are more tractable to a simulation approach.

Model 1

Figure 3.3. shows a CELLSIM program which simulates an FLM experiment on a system where cells are cycling asynchronously with the parameters shown in Table 3.1. The distributions of times spent in G_1, S and G_2 are simple trapezoidal distributions which approximately mimic typical actual distributions; the variances are relatively high, the coefficient of variation for each phase being 59%. Random variates from these distributions are used to provide realisations of phase durations for individual cells. At the start of simulation 1000 cells are placed in the phases of population 1 (which represents unlabelled cells), in a way consistent with an age distribution of cells from an exponentially growing population. Proliferation is allowed to occur normally for a warm-up time of 112 hours so that the cells may be considered to be distributed throughout the phases in a pattern consistent with the parameters of the model, and then the cells in S phase are moved to population 2 (which represents labelled cells).

Each hour subsequent to labelling, the fraction of mitoses labelled is graphed. Repeating this process with different sets of random numbers and different

```
CELL TYPES UNLB,LBLD;

STATES (1,2) G1,S,G2,M;

FLOW(1,2) G1-S(ALL),S-G2(ALL),G2-M(ALL),M-G1(ALL);

TIME IN STATES(1,2) G1 TRAP(0.2,8,24),S TRAP(0,2,8,24),
                    G2 TRAP(0.1,4,12),M 2 0.

NUMBER OF GROUPS(1,2) = 200;
MAXIMUM NUMBER OF GROUPS =800;

PROLIFERATION(1,2) M 2;

INOCULUM(1) 1000;

MOVE, AT TIME = 112, S(1) TO S(2);

REPORTS 112 HOURS;
CHANGE REPORTS, AT TIME=1, 1 HOUR;
GRAPH 100*M(2)/(M(1)+M(2));

RANDOM NUMBER SEED=328511;
SIMULATE 148 HOURS;
```

Fig. 3.3 A CELLSIM program modelling an FLM experiment on normally cycling cells, with a large degree of variation between cells in phase durations.

warm-up times allows us to produce a realisation of a typical FLM experiment, such as is shown (for 5 replicates) in Figure 3.4, which also gives the curve fitted by Gilbert's method. Table 3.1 compares the estimates of the parameters with those fed into the simulation. The estimate of the cell cycle time (T_c) is apparently significantly lower than it should be; this exemplifies a point which has been made previously (Al-Dewachi et al, 1974; Hull, 1980), namely that the standard error estimates provided by this curve-fitting method are too small.

In association with the observed labelling index, a value for which is also provided by the simulation, the FLM experiment can be used to estimate the

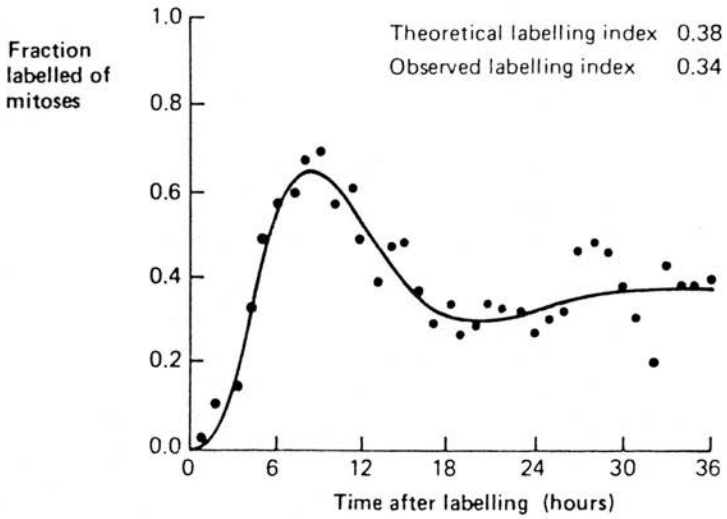

Fig. 3.4 Data from a simulated FLM experiment of a population of normally cycling cells, with a large degree of variation between cells in phase durations; and the curve fitted by Gilbert's method.

Table 3.1 Mean phase durations used by CELLSIM programs for models 1 and 2, with values estimated by Gilbert's method, and their quoted standard errors

Parameter	Actual value	Estimated value ± Standard error Model 1	Model 2
Duration of $G_1 + \frac{1}{2}M$	10.2	8.3 ± 1.2	3.1 ± 6.5
Duration of S	9.2	7.8 ± 1.0	5.2 ± 4.0
Duration of $G_2 + \frac{1}{2}M$	5.6	5.5 ± 0.4	5.5 ± 1.0
Cell cycle time	25.0	21.6 ± 1.4	13.8 ± 6.5
CV of cell cycle time	32%	$38\% \pm 3\%$	$75\% \pm 44\%$
Growth fraction	1.00	0.89	1.64

proportion of proliferating cells in a population (the growth fraction) even when there is considerable variation in phase durations (Appleton & Sunter, 1979). Since there is an error of 14% in cell cycle time estimate, it is not surprising that there should be a comparable error in growth fraction estimate. However, for this example Gilbert's method performs reasonably well, returning values which are not substantially different from the correct ones. A fuller analysis than would be appropriate here would involve many more simulations. The distinction between the program which follows the fate of the same population of cells throughout, the real FLM experiments which sample different sets of cells at each time, and the use by the program of 'groups' of cells would have to be considered more carefully; but our conclusions, though simplified, are substantially correct.

Model 2

Figure 3.5 shows a very similar CELLSIM program to that described previously. The only difference is that 12 hours before the FLM experiment was begun 80% of cells in S were killed and any cell entering S in the next 6 hours also had 80% probability of death. This naturally perturbs the age distribution of cells from

```
CELL TYPES UNLB,LBLD;

STATES (1,2) G1,S,G2,M;

FLOW(1,2) G1-S(ALL),S-G2(ALL),G2-M(ALL),M-G1(ALL);

TIME IN STATES(1,2) G1 TRAP(0.2,8.24),S TRAP(0.2,8,24),
                    G2 TRAP(0.1,4.12),M 2 0;

NUMBER OF GROUPS(1,2) = 200;
MAXIMUM NUMBER OF GROUPS =800;

PROLIFERATION(1,2) M:2;

INOCULUM(1) 1000;

KILL (1) BETWEEN 100 AND 106 S 0.8;
MOVE, AT TIME = 112, S(1) TO S(2);

REPORTS 112 HOURS;
CHANGE REPORTS, AT TIME=1, 1 HOUR;
GRAPH 100*M(2)/(M(1)+M(2));

RANDOM NUMBER SEED=328511;
SIMULATE 148 HOURS;
```

Fig. 3.5 A CELLSIM program modelling an FLM experiment on cells which have suffered a cytotoxic insult several hours previously.

that assumed by the method of analysis, and we must determine whether it is sensitive to this violation of its underlying assumptions or whether it nevertheless manages to yield satisfactory estimates of the parameters of the system. The curve produced from 5 simulations is shown as Figure 3.6, and the results are again in Table 3.1. The results depend on the initial estimates of the parameters; other initial values give visually identical curves but values for the cell cycle time several hours different.

The most noticeable aspect of the results is the large standard errors for the parameter estimates, indicating that despite the apparent similarity between the curves of Figures 3.4 and 3.6, for the latter the method of analysis is

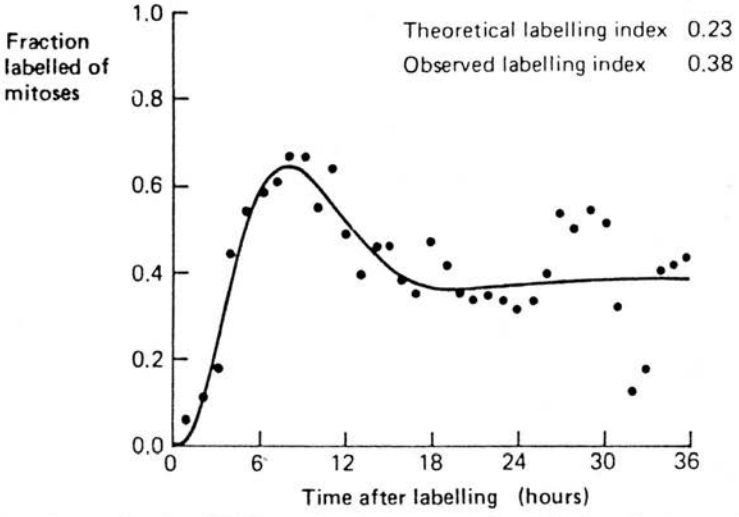

Fig. 3.6 Data from a simulated FLM experiment on a population of cells which have suffered a cytotoxic insult several hours previously.

unsatisfactory. It is, however, an aspect of Gilbert's method to be commended that it is, so to speak, sometimes capable of pointing out when it is proving an inadequate model of the system it is trying to analyse. The large standard errors give clear warning that the results are untrustworthy. Indeed the cell cycle time is underestimated by some 45% and the growth fraction estimate is ridiculous; this error is compounded by the sensitivity of the estimate to the CV of the cell cycle time. Nor is there any guarantee that an underestimate will always be obtained for the cell cycle time; the exact outcome will depend on the nature of the disturbance to the cell age distribution, how long it took place before the FLM experiment was started, and whether there are any compensatory mechanisms in operation. We have in fact assumed a very simple model where cell proliferation is completely unaffected by the insult; we must expect to be in a worse position in real life.

Model 3

Figure 3.7 shows a CELLSIM program which models a more complex cell kinetic system. The G_1 phase is divided into five sub-phases, after each of which a cell has a probability of 0.1 of entering a G_0 (or longer G_1) phase and subsequently

```
CELL TYPES  UNLB,LBLD;

STATES (1,2) G11,G12,G13,G14,G15,S,G2,M,G10,G20;

FLOW (1,2) G11 - G12 (0.9),  G11 - G10 (0.1),
           G12 - G13 (0.9),  G12 - G10 (0.1),
           G13 - G14 (0.9),  G13 - G10 (0.1),
           G14 - G15 (0.9),  G14 - G10 (0.1),
           G15 - S   (ALL),  S   - G2  (ALL),
           G2  - M   (0.9),  G2  - G20 (0.1),
           M   - G11 (ALL),
           G10 - G11 (ALL),  G20 - G2  (ALL);

TIME IN STATES (1,2)  G11: TRAP(0.1,4,12),
                      G12: TRAP(0.1,4,12),
                      G13: TRAP(0,1,4,12),
                      G14: TRAP(0,1,4,12),
                      G15: TRAP(0,1,4,12),
                      S  : TRI(2,6,16),
                      G2 : TRI(1,4, 7),
                      M  : 1,
                      G10: TRI(25, 50,150),
                      G20: TRI( 5, 10, 30);

NUMBER OF GROUPS (1,2) = 200;
MAXIMUM NUMBER OF GROUPS = 800;

PROLIFERATION (1,2) M: 2;

INOCULUM (1) G11:90, G12:65, G13:60, G14:55, G15 50, S 170,
                             G2:20, M:10, G10 450, G20 30;

MOVE, AT TIME = 200, S(1) TO S(2);

REPORTS 20 HOURS;
CHANGE REPORTS, AT TIME = 199,  1 HOUR;

GRAPH 100*(M(1)+M(2))/TOTAL;
GRAPH 100*M(2)/(M(1)+M(2));
GRAPH 100-100*(G10(1)+G10(2)+G20(1)+G20(2))/TOTAL;

RANDOM NUMBER SEED = 300944;

SIMULATE  240 HOURS;
```

Fig. 3.7 A CELLSIM program modelling an FLM experiment on cells which may enter long G_1 or G_2 phases.

restarting the cycle; there is also the possibility of entering a long G_2 phase. Figure 3.8 shows the FLM data resulting from 5 replicates, and the fitted curve.

It is clear from the graph that the curve does not fit the data well, and indeed estimates of the phase durations could only be obtained by reducing the usual 6 parameters to 5 by postulating that the coefficient of variation of $S + G_2$ was the same as that of the cell cycle time. The model is designed so that a cell which completes the cell cycle without going through one of the long phases has an expected cycle time of 36 hours and the cell cycle time corresponding to the fitted curve is 35.8 hours. However, only 59% of cells take this course and the distribution of cell cycle times has a very long positive tail with a mean value about twice as high. If a growth fraction estimate close to 0.59 were obtained this would be relatively satisfactory, but the value found is 0.93. In other words, the FLM experiment hardly detects the presence of the long cycling cells at all. It also estimates the mean duration of S as 4.8 ± 0.7 h when it is in fact 8 h. Some of the

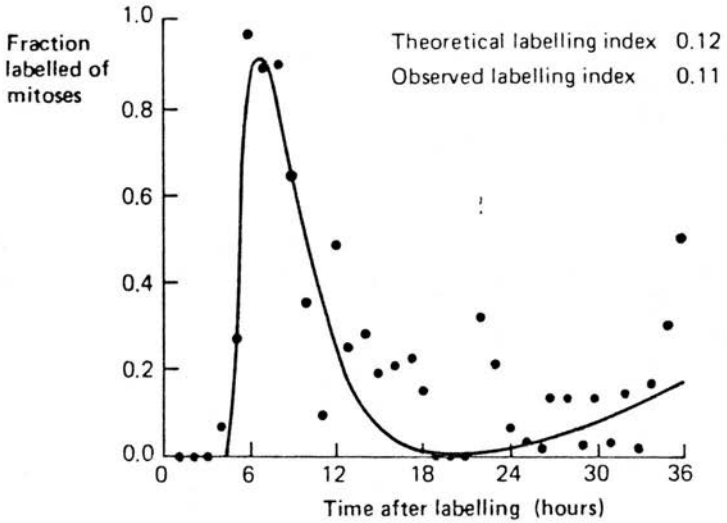

Fig. 3.8 Data from a simulated FLM experiment on a population of cells which may enter long G_1 or G_2 phases.

difficulties arise because the experiment is too short in relation to the cell cycle time, but in such a situation one must question not only whether the analysis is appropriate but also whether the FLM technique itself can give us the information we require, which is a description of the mechanisms of the system rather than simple phase durations.

DISCUSSION

The standard methods of analysing FLM experiments, whether merely based (as in Fig. 3.1) on specific points on a curve fitted by eye to the data, or on a computer model, are dependent on a number of assumptions. Some of the assumptions are not crucial, such as the precise distributions assumed for the durations of the phases of the cell cycle time, or the precise definition of the best-fitting curve (Hartmann et al, 1975). Gilbert's method, which we have studied here, as it has been our chosen method of analysing well over a hundred FLM curves in the last 10 years, has a rather odd optimisation criterion using Laplace transforms which generally leads to underestimates of standard errors of the parameters it estimates. However, as we have shown here, it copes well with high variability and when it is applied to data which violate its assumptions it is likely to indicate this either by giving very large standard errors for the estimated parameters, or by converging with difficulty, if at all, to any estimates. It is important to take these characteristics of an analytical method into account, for if the method is treated merely as a way of providing definitive values of parameters, erroneous conclusions will be reached.

So far the discussion of the FLM curve has been quite general; let us now turn to its use in psoriasis. There is certainly a high degree of variability both in S-phase (Weinstein & Frost, 1968) and in the cell cycle time as a whole (Duffill,

Wright & Shuster, 1976); it is probable that the cell cycle is more complex than the simple analytical methods assume (Goodwin, Hamilton & Fry, 1973). Thus the sort of problems we have been discussing are relevant to the study of untreated psoriasis, and any attempt to investigate the kinetics after treatment may well be further hampered by the difficulties brought about by synchrony.

There are further difficulties: although we can make educated guesses at the cell age distribution (Appleton, Wright & Dyson, 1977) we do not know it precisely, and this affects estimates of growth fractions even if such a concept is relevant. Also we must pay attention to what may be a crucial distinction between variation within an individual and variation between individuals: Weinstein and Frost (1968) pool their data from different individuals and fit a single FLM curve, while Goodwin, Hamilton and Fry (1974) fit separate curves and average the estimated parameters. The second method is to be preferred (Gore, Jones & Rytter, 1976), although it may be difficult in practice to obtain enough points for each individual, especially on the critical descending limb of the curve, to allow reasonable estimates to be calculated for each individual.

However, the main difficulty confronting the analyses of any cell kinetic experiment in psoriasis is the lack of a suitable model combining both the spatial and temporal aspects of the system to be studied. Any serious attempt to understand the proliferative mechanisms will involve a specific model (c.f. Appleton, Morley & Wright, 1973), perhaps using CELLSIM. Such use of simulation would have to be much more extensive than the simplified, didactic use presented here, but even then hopes of obtaining detailed information on modes of migration, resting phases and feedback control in the complex human condition should be guarded.

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David Appleton

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12

THE CELL CYCLE TIME IN THE RAT JEJUNAL MUCOSA

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(*Received* 21 *March* 1974; *revision received* 8 *July* 1974)

ABSTRACT

The regional variation of the duration of cell cycle parameters was studied by constructing fraction of labelled mitoses curves at several levels in the jejunal crypt column of male Wistar rats. Prolonged T_c and T_s values were apparent only in the bottom eight cell positions, and these differences were shown to be significant compared with the remaining cell positions by analysing the data by the method of Gilbert (1972). Above cell position 8 the proliferating crypt cells showed effectively the same phase durations. For the whole crypt column T_c was $11{\cdot}32 \pm 0{\cdot}14$ (SE) and T_s $6{\cdot}49 \pm 0{\cdot}10$.

Although variation in phase durations was confined to the basal portion of the crypt, the results essentially confirm the findings of Cairnie, Lamerton & Steel (1965a), and may be interpreted in terms of the slow cut-off model. The demonstration of prolonged T_c values in basal cell positions confirms the presence of a longer cycling subpopulation of cells at the bottom of the crypt.

INTRODUCTION

In terms of cell cycle parameters, the mouse small bowel has been more fully explored than that of the rat; indeed, in the rat we depend almost exclusively upon the data of Cairnie, Lamerton & Steel (1965a). Employing the fraction of labelled mitoses (FLM) technique, Cairnie *et al.* (1965a) reported a mean cell cycle time (T_c) of 10·5 hr for the rat jejunal crypt when all cell positions in the crypt were included in the computation. Furthermore, when the crypt was divided into several portions, and separate FLM curves duly constructed, differences in T_c and in component phase times became apparent. The cells at the base of the crypt showed a T_c of 14 hr, but this value became steadily shorter with progress up the crypt, and in the uppermost portion was reduced to 10 hr; the decrease was apparently due to changes in T_s and T_{g1} (the duration of DNA synthesis and of the presynthetic interval, respectively).

Independent measurements of T_c in the rat using stathmokinetic techniques (Tannock, 1967; Wright, Morley & Appleton, 1972) have largely confirmed the value for the whole crypt. Recently, however, Tutton (1973) has claimed (also on the basis of stathmokinetic

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techniques) that T_c in the bottom ten cell positions of the rat crypt is much longer, of the order of 26 hr in the jejunum. Tutton maintained that computer simulation of the FLM results of Cairnie *et al.* (1965a), using the methods of Steel & Hanes (1971), or of Gilbert (1972), would 'almost certainly lead to longer estimates of cell cycle time'.

This leads to another important point concerning the nature of the differences in cell cycle parameters between the various portions of the crypt (Cairnie *et al.*, 1965a). Baserga & Malamud (1969) have rightly insisted on caution in claiming differences between cycle parameters (as measured from FLM curves) in the absence of statistical analysis. It must be admitted that some of the differences shown between the various portions of the crypt are small; for example, between positions 7 and 9 T_c was 11·5 hr, and above position 22 10 hr, a decrease of 1·5 hr (13%). Application of a computer simulation technique would be expected to cast some light on the importance of these differences.

Wright *et al.* (1972) reported differences in the duration of mitosis (T_m) with cell position in the crypt; T_m appeared to increase as the uppermost cell positions were reached. Such an increase would not be consistent with a general decrease in the duration of cycle parameters with ascending cell position in the crypt.

In order to investigate these several problems, we have attempted to confirm the results of Cairnie *et al.* (1965a) in the jejunal crypt of the male Wistar rat, for which detailed stathmokinetic data have already been published (Wright *et al.*, 1972). We have analysed our FLM data using the method of Gilbert (1972) for computer simulation of FLM curves, and have also applied the Gilbert analysis to the data of Cairnie *et al.* (1965a) to allow comparison of the two sets of results.

MATERIALS AND METHODS

Male albino Wistar rats aged 3 months and weighing 150–200 g were used throughout. All injection procedures were carried out at 09.00 hours. Forty-one animals were given tritiated thymidine, ^{3}H-TdR (Radiochemical Centre, Amersham, England) in a dosage of 0·5 μCi per g body weight, by intraperitoneal injection. The concentration used was 100 μCi/ml. For the first 15 hr after ^{3}H-TdR injection animals were killed by cervical dislocation at intervals of 30 min, and thereafter every hour up to 27 hr.

Portions of jejunum were excised from the region immediately below the ligament of Treitz, fixed in Carnoy's fluid for 6 hr, and embedded in paraffin. Serial transverse sections were cut at a thickness of 3 μm, and autoradiographs prepared using Kodak A.R. 10 stripping plates. The autoradiographs were developed with Kodak D 19 b, and then fixed, washed, and stained through the emulsion with Harris's haematoxylin.

Axially sectioned crypts were selected, i.e. crypts sectioned so that the base, middle and top of the crypt were all in the plane of section. The left-hand column of cells (the crypt column) was divided into six portions consisting respectively of cell positions 1–4, 5–8, 9–12, 13–16, 17–20, and those cell positions above position 20, The mitotic index varies with cell position in the crypt (Cairnie *et al.*, 1965a; Wright *et al.*, 1972), with highest indices appearing between cell positions 6 and 20. Mitoses were counted until at least twenty had been scored for cell positions 1–4 and those positions above 20. Mitoses were of course more abundant in the other cell positions, where at least 100 mitoses were counted in each group. A differential count of labelled and unlabelled metaphases and anaphases was made, and the fraction of labelled mitoses plotted against time after injection of ^{3}H-TdR. Only metaphases and ana-

phases were recorded in order to overcome the difficulty experienced in recognizing prophases and telophases in sections.

The data were analysed by the method of Gilbert (1972), who gives full details of the technique. Gilbert proposed that when gamma distributions are used to describe the durations of the phases of the cell cycle, the Laplace transform of an FLM curve can be described by simple analytical functions. Briefly, to find the cycle parameters which best fit a set of data, a Laplace transform is obtained by integrating the data numerically for values of the angular frequency. Then by a least squares method the optimum theoretical Laplace transform curve is found using these new observational values. The theoretical FLM curve with time is generated by numerical integration of an inverse transform. The analysis is best carried out on a computer and the program was written in ALGOL W and run on an IBM 360/67 computer. The method generates mean values for the time parameters of the cell cycle with standard errors, and coefficients of variation (CV), also with standard errors.

RESULTS

The FLM curves for the different portions of the crypt are shown in Fig. 1. The experimental values are denoted by the solid points, while the open circles and the dotted line represent the line generated by the Gilbert program.

In the case of the FLM curve for the whole crypt (Fig. 1a), the computed curve fits the data points well over the first two peaks; however, the computed curve, though apparently showing a third peak, does not follow the experimental points into the second trough. Taken as a whole, however, the fit is good and the discrepancies may be explained (see Discussion). The FLM curve for cell positions 1–4 (Fig. 1b) shows a good deal of damping compared with the curves at higher cell positions, and only completed two peaks in the 27 hr experimental period. The damping is due to variation in the cell cycle time, and denotes a wider spread of individual cell cycle times at different cell positions compared with the higher cell positions. The fit over the first peak is good; in the second peak the computed curve may rise slightly sooner than would be expected from the data points, but again the fit is generally good. The curve for cell positions 5–8 (Fig. 1c) is not nearly so damped as the previous curve, and describes two clear peaks with the beginning of a third. In this case the computed curve fits the data points well over the first two peaks, but as in the case of the whole crypt (Fig. 1a) the fit in the second trough is not as good.

The curves for the remainder of the crypt are basically similar and may be considered together. There is very little damping shown in any of the curves, with the second peak reaching at least 0·8 in each case, These curves all show two well-defined peaks, with the initial portion of a third peak well described, and the appearances suggest that in these positions there is little spread in T_c or in component phase durations either between or within different cell positions. In each curve the data points in the first peak, first trough and second peak are well fitted by the computed line, and although a third peak is clearly depicted, the second trough is again not followed. As Gilbert (1972) points out, the standard errors generated by his program should not be used as the basis of significance tests. Our experience with simulated data indicates that these standard errors are too small by a factor of 2 or 3 in this situation, and we have chosen to use the terms 'significant' and 'highly significant' when differences are more than 6 or 8 times the larger standard error involved. We do not claim that the criteria are equivalent to the conventional 5% and 1% probability levels, but they are sufficiently close for the terms not to lead to confusion.

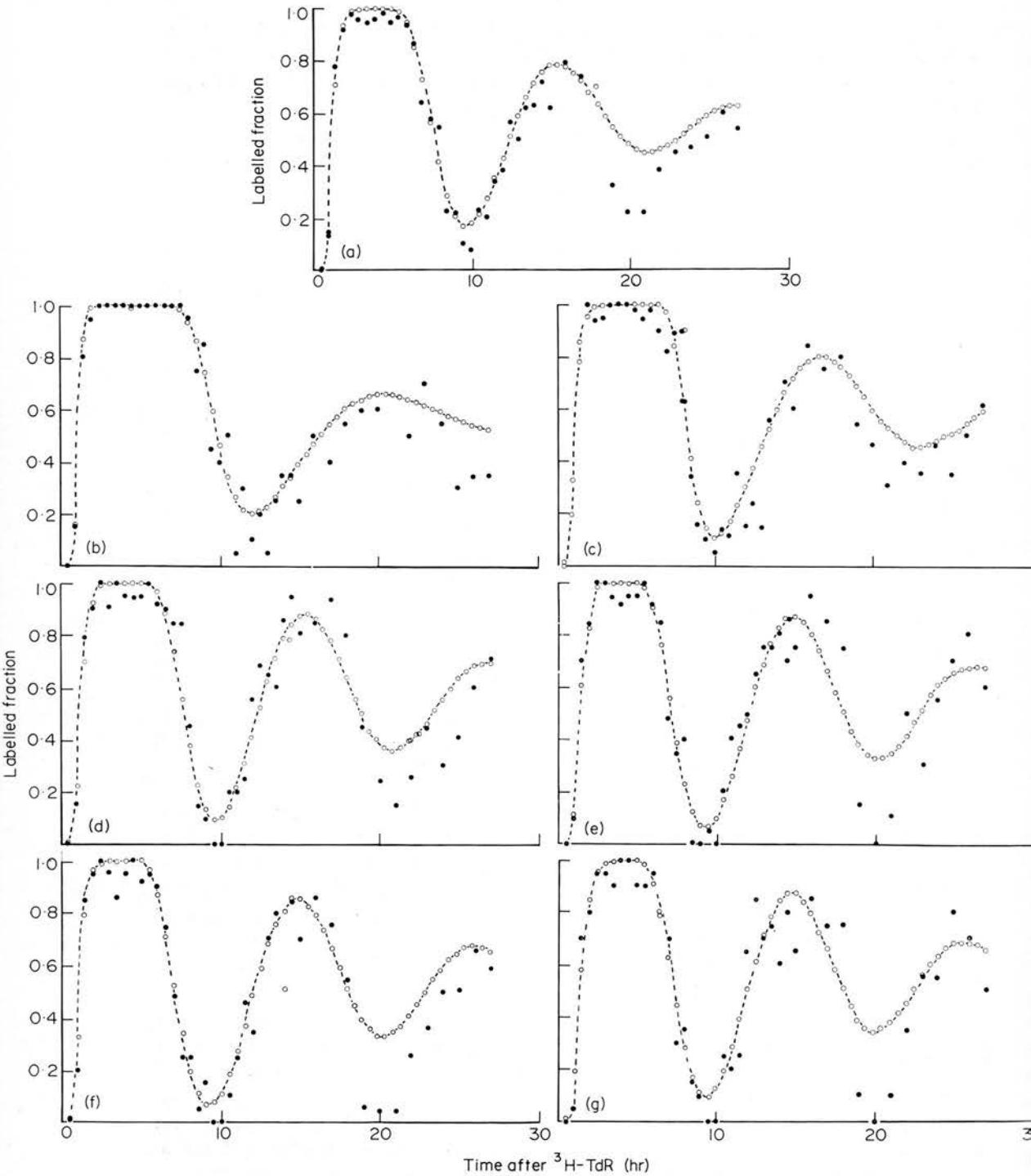

FIG. 1. FLM (fraction of labelled mitoses curves) for various portions of the rat jejunal crypt. The solid points represent the observational values, while the open circles and dotted line represent the curve computed by the Gilbert program. (a) Whole crypt; (b) cell positions 1–4; (c) cell positions 5–8; (d) cell positions 9–12; (e) cell positions 13–16; (f) cell positions 17–20; (g) cell positions above 20.

The mean values for the duration of the phases of the cell cycle, together with standard errors (SE) and coefficients of variation (CV) with standard errors, are given in Table 1. For the whole crypt T_c is 11·32 hr ± 0·14 (SE) and T_s is 6·49 hr ± 0·10. It should be remembered that the Gilbert program regards mitosis as an instantaneous event, and this will affect the

TABLE 1. Mean values of the durations of the phases of the cell cycle in different portions of the jejunal crypts of male Wistar rats

	T_c	T_{g1}	T_s	T_{g2}	CV_c	CV_{g2}	CV_{s+g2}
Cell positions 1–4							
Mean	15·48	5·70	8·56	1·23	30·50	18·67	11·99
SE	0·24	0·24	0·10	0·04	1·65	15·34	1·64
Cell positions 5–8							
Mean	12·34	4·00	7·14	1·21	21·61	34·23	8·23
SE	0·14	0·16	0·08	0·04	1·16	8·49	1·47
Cell positions 9–12							
Mean	11·24	3·50	6·42	1·32	15·99	31·65	13·25
SE	0·14	0·20	0·14	0·06	1·56	10·62	2·47
Cell positions 13–16							
Mean	10·82	3·55	5·85	1·43	15·52	25·94	13·49
SE	0·14	0·19	0·13	0·06	1·52	9·50	2·16
Cell positions 17–20							
Mean	11·03	3·89	5·94	1·20	16·16	31·96	13·77
SE	0·15	0·20	0·14	0·06	1·55	11·95	2·50
Cell positions above 20							
Mean	10·66	3·24	5·96	1·46	15·42	36·18	14·18
SE	0·12	0·17	0·12	0·06	1·42	8·08	2·21
Whole crypt							
Mean	11·32	3·52	6·49	1·30	21·00	31·75	15·14
SE	0·14	0·16	0·10	0·04	1·33	7·23	1·72

computed values of T_{g1} and T_{g2}. At the bottom of the crypt column T_c in cell positions 1–4 ($T_{c(1-4)}$) is 15·48 hr ± 0·24. This value is more than 12 SE different from $T_{c(5-8)}$, in the immediately superior cell positions, and the difference is considered highly significant. The CV at 30·50% is comparatively large. This confirms that a considerable spread in individual values of T_c is to be expected in this portion of the crypt. T_s in these cell positions is 8·56 hr ± 0·1, which is significantly larger than $T_{s(5-8)}$. We conclude that the cells in the lowest cell positions are cycling comparatively slowly, and show significantly longer T_c and T_s values compared with higher cell positions.

$T_{c(5-8)}$ is 12·34 hr ± 0·14 and $T_{s(5-8)}$ 7·14 hr ± 0·08. Here $T_{c(5-8)}$ is significantly longer than $T_{c(9-12)}$. $T_{s(5-8)}$ in only five standard errors longer than $T_{s(9-12)}$, though $T_{s(13-16)}$ is 5·85 hr ± 0·13 and when compared with this, $T_{s(5-8)}$ is over nine standard errors longer. We conclude that in these cell positions, $T_{c(5-8)}$, although shorter than $T_{c(1-4)}$, is significantly longer than in higher cell positions in the crypt column. T_s appears to shorten with increasing cell position, as $T_{s(13-16)}$ is significantly shorter than $T_{s(5-8)}$. It should also be noted that CV_{s+g2} remains small at all cell positions studied, a consequence of the narrow distribution of T_s values.

The cycle parameters in the remaining cell positions do not differ significantly; T_c is close to 11 hr and T_s close to 6 hr. The CV_c values for the upper four groups are comparatively low, emphasizing the narrower distribution of cycle times towards the top of the crypt.

We conclude that the cell cycle time in the lower cell positions is significantly longer than in remaining cell positions, but that this difference is confined to the bottom two groups studied. Above these, crypt cells are cycling with effectively the same T_c. Similar comments also apply to T_s, although this parameter would appear to change more slowly.

DISCUSSION

The mean cell cycle time for the whole crypt (11·32 hr) is some five standard errors different from the mean T_c of 10·59 hr here computed from the data of Cairnie *et al.* (1965a) and shown in Table 2: allowing for strain differences, the agreement is good. Wright *et al.* (1972), employing a stathmokinetic technique with vincristine, reported a T_c value of 10·4 hr in male Wistar rats, while Tannock (1967) obtained a value of 11 ± 1 hr in male August rats, also using vincristine. Tutton (1973) has found much longer values in the lower 10 cell positions of jejunal crypts of male Sprague-Dawley rats; for example, a stathmokinetic technique carried out between 06.00 and 10.00 hours (most closely approximating to the time used by Tannock (1967) and Wright *et al.* (1972) gave a T_c of 26·6 hr, more than double the stathmokinetic and FLM values discussed above for the whole crypt. Admittedly the FLM results were obtained over a longer time period (for a median T_c effectively 09.00–20.00 hours), while during this period Tutton found diurnal changes in T_c from 26·6 hr to 14·5 hr; an intermediate T_c measured between 12·00 and 16.00 hours was 17.1 hr. It is of course possible that T_c for total proliferating crypt cells changes in the same way; the similarity between the T_c values of Cairnie *et al.* (1965a) and of Tannock (1967) for August rats, and between Wright *et al.* (1972) and the present results, suggest that any such changes are small, since the agreement between a short stathmokinetic experiment and the longer FLM technique is good. We also conclude that when mean values for T_c are calculated from the data of Cairnie *et al.* (1965a) using the Gilbert program, there is no evidence of the prolongation expected by Tutton (1973) (see Table 2), at least in the two strains considered.

Our results show a prolongation of T_c in the two basal portions of the crypt studied, but above cell position 8 there was no change in T_c. Our analysis of the data of Cairnie *et al.* (Table 2) allows a similar conclusion (see Table 2).

These conclusions concerning longer cycling cells at the base of the crypt are especially interesting; Cairnie *et al.* (1965a) speculated that T_c values as long as 50 hr may be apparent at cell position 1, and Wright *et al.* (1972) provided additional evidence by calculating apparent cell cycle times ($T_{c(a)}$) for the lower four cell positions after excluding Paneth cells. Assuming exponential growth conditions and a growth fraction of unity at these positions, T_c is 30 hr, 22 hr, 15 hr and 10 hr in cell positions 1–4 respectively, calculated from the rate of entry into mitosis measured with vincristine. The mean T_c obtained for these positions from the FLM data (Table 1) is 15·48 hr. The FLM technique would be expected to emphasize those cell positions with highest mitotic indices and appears to be consistent with the stathmokinetic data: this may justify the assumption of a growth fraction of unity, since the FLM technique is independent of growth fraction considerations. There is now little doubt that these basal cells do have a prolonged T_c, corroborating Lamerton's (1972) contention that the earliest stem cells found in the crypt are slowly cycling.

TABLE 2. Mean values for the duration of the phases of the cell cycle in different positions of the jejunal crypts of male August rats, calculated from the data of Cairnie *et al.* (1965a)

	T_c	T_{g1}	T_s	T_{g2}	CV_c	CV_{g2}	CV_{s+g2}
Cell positions 1–6							
Mean	13·87	4·52	8·11	1·24	23·62	27·96	19·23
SE	0·24	0·26	0·15	0·04	1·85	8·16	2·16
Median*	14·0	3·5	8·5	1·0			
Cell positions 7–9							
Mean	11·89	3·55	6·77	1·57	22·29	39·22	14·93
SE	0·22	0·25	0·14	0·06	2·00	7·79	2·24
Median*	11·5	2·5	7·0	1·0			
Cell positions 10–12							
Mean	10·70	2·84	6·53	1·33	17·95	51·56	10·30
SE	0·15	0·19	0·13	0·07	1·70	8·38	2·16
Median	11·0	2·5	6·5	1·0			
Cell positions 13–15							
Mean	10·44	2·66	6·54	1·24	18·06	49·64	15·10
SE	0·12	0·14	0·11	0·05	1·42	6·65	1·91
Median	10·5	2·0	6·5	1·0			
Cell positions 16–18							
Mean	10·06	1·92	6·73	1·40	15·27	53·10	21·83
SE	0·22	0·21	0·25	0·08	2·38	9·89	4·04
Median*	10·0	1·5	6·5	1·0			
Cell positions 19–21							
Mean	9·98	2·22	6·36	1·39	16·40	42·02	19·37
SE	0·12	0·14	0·13	0·05	1·45	6·57	2·45
Median*	10·0	1·5	6·5	1·0			
Cell positions above 22							
Mean	10·60	3·08	6·25	1·27	19·85	45·38	10·65
SE	0·20	0·24	0·14	0·07	1·96	9·43	2·53
Median*	10·00	1·5	6·5	1·0			
Whole crypt							
Mean	10·59	2·82	6·43	1·34	22·91	42·43	12·27
SE	0·14	0·15	0·09	0·04	1·46	5·93	1·65
Median*	10·5	2·0	6·5	1·0			

* As read from the 50% peak values (Cairnie *et al.*, 1965a).

The T_c values reported by Tutton (1973) are long when compared with the present results. $T_{c(a)}$ calculated by pooling the stathmokinetic data for the first ten cell positions of the Wistar rat, and assuming exponential conditions, is 12 hr, compared with a value of 26 hr found by Tutton for approximately the same time of day in Sprague-Dawley rats. These values are, of course, apparent cell cycle times, and depend on the assumption of a growth fraction of unity; this assumption would appear to be vindicated by our data, as T_c calculated for cell positions 1–8 from the present FLM data is 13·3 hr, and $T_{c(1-9)}$ from the data of Cairnie *et al.* (1965a) is also 13·3 hr. One factor which may account for the longer times obtained by Tutton is the possibility that metaphase degeneration occurred over the relatively prolonged metaphase arrest period of 4 hr (Aherne & Camplejohn, 1972). Regional variation in T_m in the crypt was reported by Wright *et al.* (1972); longest values were found in the basal

cell positions, while shortest values were evident between positions 4 and 19. These observations are consistent with the changes in T_c which have here been demonstrated. However, they also claimed a further prolongation of T_m in cell positions above 20; this point is not supported by the present data, as the FLM results show no lengthening of T_c in this region of the crypt, and an increase in T_m while T_c remains constant would be an unusual situation in physiological conditions.

The FLM curves obtained for the various groups of cells show considerable variation, from the heavily damped curve of positions 1–4 to the near square wave form of the curve for cell positions above 20. The Gilbert program generally fitted the data points very well, with the exception of the second trough. Possible reasons for this discrepancy include errors due to decreasing grain count, and the inherent emphasis placed by the program on the early experimental values.

Our results largely confirm those of Cairnie *et al.* (1965a) regarding both the cell cycle time for the whole crypt, and the regional variation in the duration of cell cycle parameters. We could not, however, confirm a gradual decrease in T_c with increasing cell position in the crypt, finding instead that all proliferating cells above cell position 8 in the crypt column show effectively the same phase durations. But T_c in the bottom cell positions and particularly in positions 1–4, was shown to be significantly prolonged compared with higher cell positions. The data also confirm that the fall in proliferative indices noted towards the top of the crypt column in labelling and mitotic index distribution curves (Cairnie *et al.*, 1965a; Wright *et al.*, 1972) is not due to a prolongation of T_c, as previously suggested by Quastler *et al.* (1958). The existence, towards the top of the crypt, of constant T_c and T_s values with a falling labelling index, is instead fully consistent with the slow cut-off model (Cairnie, Lamerton & Steel, 1965b).

ACKNOWLEDGMENT

This work was supported by a grant from the North of England Council of the Cancer Research Campaign.

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13

CELL POPULATION KINETICS IN THE RAT JEJUNAL CRYPT

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(*Received* 1 *July* 1974; *revision received* 6 *February* 1975)

ABSTRACT

Cell kinetics in the jejunal crypt of the male Wistar rat were studied using autoradiographic techniques with tritiated thymidine and a stathmokinetic technique with vincristine. The migration rate measured by following the movement of the 50% peak on the labelling index distribution curve with time after injection of tritiated thymidine gave a value of 1·43 ± 0·14 (SE) cell positions per hour, compared with a value from a cumulative birth rate of 1·78 cell positions per hour.

The crypt column length was 32·9 ± 0·2 cells and the column count was 22·3 ± 0·2. This measurement gave a total crypt population of 734 cells, compared with an estimate of 650 ± 6 from direct observation of squashed, microdissected crypts. In each crypt 22·5 ± 0·5 mitoses were present, and the crypt cell production rate was 32 cells per crypt per hour; this latter value was confirmed using two independent techniques.

The crypt growth fraction calculated from the durations of phases of the cell cycle and the labelling index was 0·62. A value of 0·61 was found from the labelling index distribution curve. As assessed from crypt squashes, there were 403 proliferating cells per crypt.

INTRODUCTION

Considerable progress has been made towards a kinetic definition of the small bowel crypt in terms of size, flux and time, particularly in the mouse (Hagemann, Sigdestad & Lesher, 1970; Kovacs & Potten, 1973). In the rat, however, fewer results are available, particularly for the growth fraction and the crypt cell production rate; indeed, for the latter parameter, we rely almost exclusively on the results of Clarke (1971), obtained by metaphase arrest in microdissected crypts.

It is the purpose of this communication to present values for several kinetic parameters in the rat small bowel crypt, including the crypt cell production rate, and employing alternative techniques.

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MATERIALS AND METHODS

Three-month-old male Wistar rats weighing 150–200 g were used throughout. All injection procedures were carried out at 09.00 hours in an attempt to minimize the effects of diurnal variation (Sigdestad, Bauman & Lesher, 1969).

Experiment 1

Rats were given 0·5 μCi per g body weight of tritiated thymidine, ^{3}H-TdR (Radiochemical Centre, Amersham), by intra-peritoneal injection. The animals were killed at intervals thereafter up to a period of 9 hr. Samples of small bowel were removed at a site immediately distal to the ligament of Treitz and were fixed in Carnoys fluid for 6 hr. Paraffin sections were cut at a thickness of 3 μm, and autoradiographs were prepared as previously described, employing an exposure period of 28 days (Al-dewachi *et al.*, 1974).

In each animal 100 axially sectioned crypts were selected and the distribution of labelled cells in the left-hand crypt column was recorded, counting from the bottom upwards to the crypt–villus junction. The variation in the distribution of labelled cells as between short and long crypts (Cairnie & Bentley, 1967) was compensated for as previously described (Wright, Morley & Appleton, 1972a, b), and crypt labelling index distribution curves were constructed for each animal.

On each labelling index distribution curve the position, on the trailing edge, of 50% of the maximum or peak value was found and the corresponding cell position noted. This value was then plotted against time after ^{3}H-TdR injection. The slope of the line so attained is a measure of cell velocity towards the top of the crypt (Cairnie, Lamerton & Steel, 1965).

Experiment 2

The details of this stathmokinetic experiment with vincristine are given in a previous paper (Wright *et al.*, 1972a).

Experiment 3

Three animals were killed at 09.00 hours, and samples of upper jejunum were prepared for crypt microdissection following the procedure of Clarke (1971). After perfusion *in situ* with ethanol acetic acid (3:1) the whole small bowel was fixed in the same mixture for 24 hr and stored in 75% ethanol. Small portions of upper jejunum were brought to water. After hydrolysis at 60°C for 6 min the tissue was immersed in Schiff's reagent for 1 hr, washed and transferred to 45% acetic acid. Ten crypts were dissected from each animal and squashed between a slide and cover slip. A count was made of the total number of cells per crypt, and the total number of mitoses per crypt was also noted.

RESULTS

The labelling index distribution curve

Fig. 1 shows the labelling index (I_S) distribution curve for the combined values from three animals killed at 1 hr after ^{3}H-TdR. Lower values are apparent in the lower cell positions,

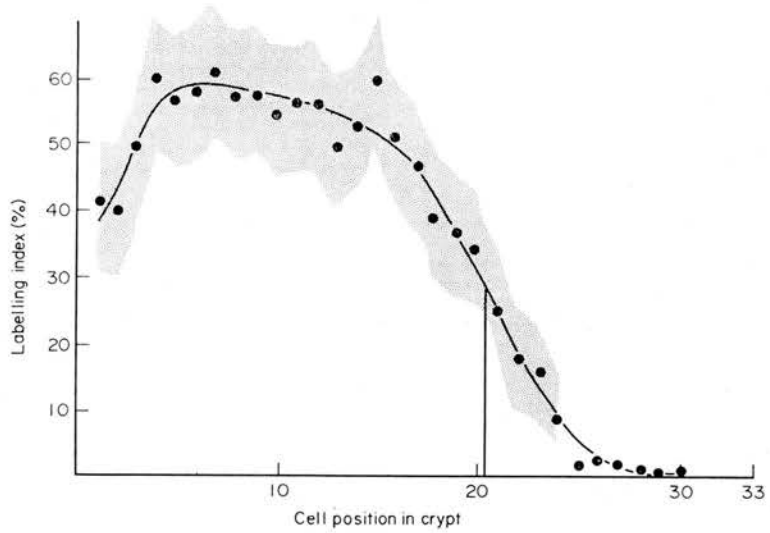

FIG. 1. The 1 hr labelling index distribution curve for the rat jejunal crypt. The perpendicular is drawn at the cell position of the 50% peak value, and the shaded area indicates 95% confidence limits for the points.

but the I_S then quickly rises to peak values of almost 60% after cell position 4. These high values are maintained until cell position 15, when a sharp fall occurs, with indices below 10% above cell position 24.

In Fig. 2 the labelling index distribution curve is compared with the mitotic index distribution curve (Wright *et al.*, 1972a). As noted before by Cairnie *et al.* (1965), the labelling index falls before the mitotic index, and mitoses are found at a higher position than labelled cells. There are also four cell positions (on the standardized curve) at the top of the crypt, where mitoses are not found.

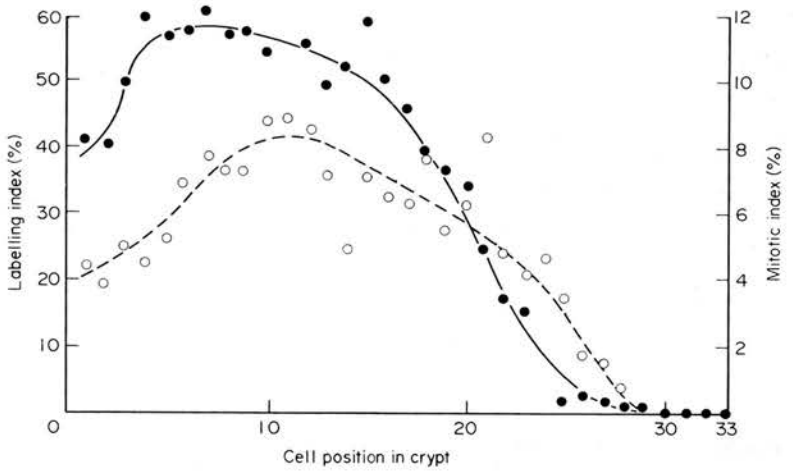

FIG. 2. The labelling index distribution curve (●, and solid line) compared with the mitotic index distribution curve (○, and dashed line).

The migration rate

Fig. 3 shows the movement with time of the 50% peak value for 9 hr after administration of ^{3}H-TdR. After 9 hr labelled cells begin to appear on the villi, and although the 50% peak

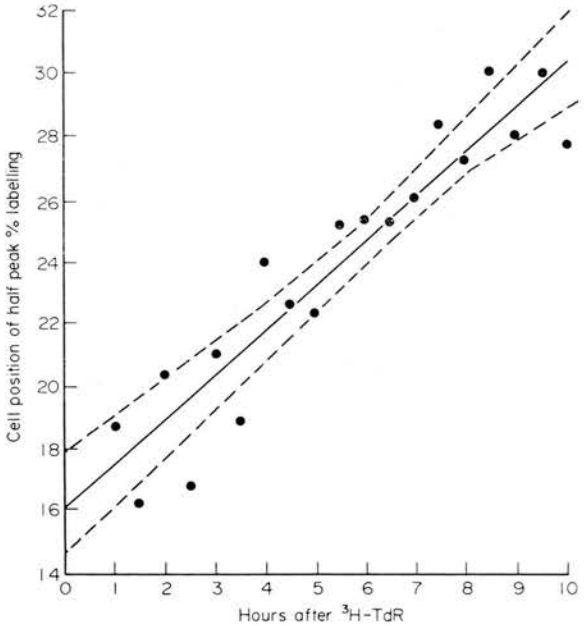

FIG. 3. Movement of the 50% peak value on the labelling index distribution curve with time after ^{3}H-TdR. The broken lines indicate 95% confidence limits for the line.

has not yet reached the crypt–villus junction, the loss of labelled cells onto the villi would render inaccurate a crypt labelling index distribution diagram drawn after this time. The line was fitted by the least squares method, and the dotted lines are 95% confidence limits for the line. A good degree of linearity is apparent, with an r^2 value of 0·96. The slope of the line is a measure of the cell migration rate, expressed in cell positions per hour, and is equal to 1·43 ± 0·14 (standard error).

The method of constructing cumulative birth rate curves using T_m values measured for each cell position has already been described (Wright *et al.*, 1972a) and Fig. 4 shows the results of a further experiment using the same technique; here the individual birth rates for each cell position have been calculated incorporating Tannock's constant (Tannock, 1967). Assuming that movement of a cell at any cell position within the crypt is due only to proliferative activity in the lower cell positions, then the maximum value reached in the cumulative birth rate curve is also a measure of cell velocity at the top of the crypt (Cairnie *et al.*, 1965a). This value was 1·78 positions per hour.

Morphometric parameters

The mean number of cells in the crypt column was 32·9 ± 0·2 (SE). A measurement was also made of the column count (Cairnie, 1967) by counting 100 approximately circular crypt

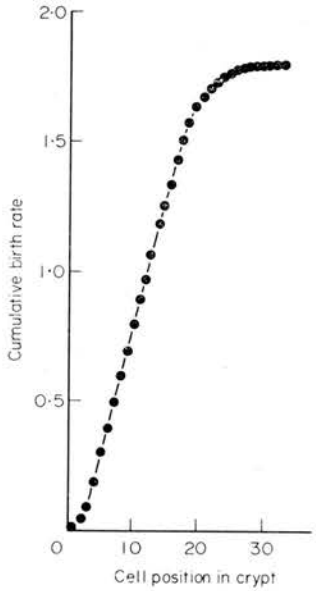

FIG. 4. The cumulative birth rate curve for the rat jejunal crypt.

cross sections, in material from three animals sectioned tangentially to the bowel lumen; the mean value was 22·3 ± 0·3. The product of the crypt column length and the column count gives an estimate of the total crypt population (Wimber & Lamerton, 1963), and here it is 734 cells per crypt.

The number of cells counted in crypt squashes was 650 ± 6 and the mean number of mitos per crypt was 22·5 ± 0·5 (3·46 ± 0·08%).

DISCUSSION

Growth fraction

The growth fraction (I_p) may be calculated for the whole crypt from the equation

$$I_p = I_{s\ expt}/I_{s\ Theor.} \quad (1)$$

where $I_{s\,expt}$ is the observed labelling index, and $I_{s\,theor.}$ is the theoretical labelling index calculated from the ratio T_s/T_c. Using the results of Al-dewachi *et al.* (1974) for T_s and T_c, and assuming steady-state conditions, a value for I_p of 0·62 is apparent.

Cleaver (1967) proposed that I_p could also be calculated from the cell position of the 50% peak value on the crypt labelling index distribution curve, when compared with the length of the whole crypt column. This method gave a similar value of 0·60.

Compartmental sizes

The total crypt population as assessed by crypt squashes was 650 cells, compared with a higher estimate of 734 cells from the product of the crypt column length and the column count.

The number of proliferating cells in the crypt may be found from the product of the crypt

population and the growth fraction; 403 from the crypt squash measurement, and 455 from the sectioned material. Non-proliferating cells therefore number 247 and 279 respectively.

Flux parameters

(a) *Crypt cell production rate and proliferative compartment efflux.* Because the maturation compartment at the top of the crypt is a simple transit compartment, any value obtained for the efflux from the proliferative compartment will also be applicable to the crypt cell production rate. The present data allow three estimates of proliferative compartment efflux.

(i) From the crypt squash data, a mean value of 22·5 mitoses was apparent in a crypt of mean size 650 cells, a mitotic index of 3·46%. The mitotic duration for the whole crypt as measured by a stathmokinetic technique was 0·43 hr (Wright *et al.*, 1972a). Now the birth rate k_b for the proliferative compartment is

$$k_b = I_m/T_m \tag{2}$$

assuming steady-state conditions; by definition all cells are proliferative. This is expressed in cells/cell/hr, and to find efflux from the total proliferative compartment we must multiply by the number of proliferative cells (403), and

$$\text{Proliferative compartment efflux} = (0{\cdot}0346/0{\cdot}43) \times 403 = 32{\cdot}4 \text{ cells/hr}$$

(ii) A value for the proliferative compartment efflux can also be adduced from T_c for the whole crypt. Assuming steady-state conditions, and a growth fraction of unity

$$k_b = 1/T_c \tag{3}$$

But I_p is less than unity for the whole crypt, and therefore

$$k_b = I_p/T_c \tag{4}$$

expressed in cells/cell/hr. Consequently the proliferative compartment efflux is

$$(0{\cdot}62/11{\cdot}32) \times 650 = 35{\cdot}6 \text{ cells/hr}$$

for a T_c of 11·32 hr (Al-dewachi *et al.*, 1974). These two values are obviously in good agreement and both compare well with Clarke's (1971) value of 35 cells/crypt/hr.

(iii) The maximum birth rate attained in the cumulative birth rate curve also equals the efflux per crypt column. The product of this value (1·78) with the number of crypt columns in the crypt (22·3) will give a value for proliferative compartment efflux, and is 39·7 cells/hr. This value, although close to the above results, is dependent upon a rigid circular spatial configuration throughout the crypt.

(b) *Transit times.* If cell migration in the crypt is a function of proliferative activity, then the cumulative birth rate at any cell position is equal to the velocity of cells at that position, expressed in cell positions traversed per hour. Consequently the time taken to traverse any cell position is the reciprocal of the birth rate at that position, and summation of these times will give the transit time through any desired portion of the crypt. Because cell-cycle times in basal cell positions are long, mitotic indices and consequently birth rates here are low (Wright *et al.*, 1972a; Al-dewachi *et al.*, 1974). These basal cells may be stem cells for the above proliferative compartment, and thus have no transit time *per se*, only a turnover rate. If these are excluded, a transit time of 34·1 hr is found for cell positions 4–33.

Since the lower limit of the maturation compartment cannot be easily fixed, it is difficult

to measure separate transit times for this compartment. However, subtraction of the mean time between incorporating ^{3}H-TdR and completing mitosis (5·0 hr) from the time taken for the 50% peak to reach the crypt–villus junction (11·2 hr), gives the transit time for a non-proliferative cell through the maturation sequence after completing mitosis (Cairnie *et al.*, 1965). This value is 6·2 hr.

(c) *Number of transit divisions.* The number of transit divisions in the proliferative compartment is a critical kinetic parameter in the control of crypt cell production. Movement of the cut-off position can control the crypt growth fraction (Cairnie, 1967; Wright *et al.*, 1972b); upward movement of the cut-off position in the absence of changes in cell cycle time (Wright *et al.*, 1972b) increases proliferative compartment size, and therefore transit time, and here the mechanism of increased cell production is the increase in the number of transit divisions (*d*) in the proliferative compartment. The present value for proliferative compartment transit

TABLE 1. A summary of kinetic parameters in the jejunal crypt of the male Wistar rat

Crypt length (cells ± SE)	Column count (cells ± SE)	Total crypt population (cells)	Growth fraction	No. of proliferating cells	No. of nonproliferative cells
32·9 ± 0·2	22·3 ± 0·3	734[a]	0·62[c]	455[e]	247[e]
		650 ± 6[b]	0·61[d]	403[f]	279[f]

Migration rate (cell positions/hr)	Crypt cell production rate (cells/crypt/hr)	Crypt transit time (hr)	Maturation transit time (hr)
1·78[g]	32·4[i]	34·1[l]	6·2[m]
1·43 ± 0·14[h]	35·6[j]		
	39·7[k]		

No. of transit divisions	Mitoses per crypt
3[n]	22·5 ± 0·5
4[o]	(3·46 ± 0·08)[p]

(a) From the product of crypt length and column count.
(b) From microdissected, squashed crypts ± SE.
(c) Using the ratio $I_{s\ expt}/I_{s\ Theor}$; $I_{s\ Theor} = T_s/T_c$.
(d) From 50% peak point on I_s distribution curve.
(e) Product of (a) and growth fraction (c).
(f) Product of (b) and growth fraction (c).
(g) Maximum attained on cumulative birth rate curve.
(h) From slope of regression line of movement of 50% peaks (Fig. 3).
(i) Birth rate for proliferative compartment calculated from I_m and T_m for whole crypt (see Discussion).
(j) Birth rate for proliferative compartment calculated from T_c and I_p (c) (see Discussion).
(k) From the product of the migration rate (h) and the column count.
(l) Calculated from the cumulative birth rate curve.
(m) From extrapolation of regression line of movement of 50% peaks.
(n) Calculated from T_c for the whole crypt and transit time for cell positions 4–29.
(o) From Cleaver's (1967) equation
(p) From crypt squashes.

time of about 31 hr suggests that, with a mean T_c of about 11 hr, some three transit divisions occur between cell positions 4 and 29.

Cleaver (1967) deduced a relationship between d and flux parameters for a proliferative compartment with no cell death.

$$d = 3{\cdot}322 \log_{10} k_{out}/k_{in} \quad (5)$$

which becomes, within the crypt column, and assuming that the basal four cell positions are stem cells,

$$d = 3{\cdot}322 \log_{10} (1{\cdot}78/0{\cdot}097) \sim \text{four transit divisions}$$

If this value were applicable, for a mean T_c of 11 hr the transit time through the proliferative compartment would be at least 44 hr which is longer than the present estimate. A summary of all the measurements is given in Table 1.

ACKNOWLEDGMENTS

This work was supported by a grant from the North of England Council of the Cancer Campaign.

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Reprinted from
Cell and Tissue Kinetics
Vol. 5, No. 4, July 1972

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD LONDON EDINBURGH MELBOURNE

Cell Tissue Kinet. (1972) **5**, 351–364.

VARIATION IN THE DURATION OF MITOSIS IN THE CRYPTS OF LIEBERKUHN OF THE RAT; A CYTOKINETIC STUDY USING VINCRISTINE

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(*Received* 4 *February* 1972; *revision received* 28 *March* 1972)

ABSTRACT

The variation in the duration of mitosis (t_m) with cell position in the small intestinal crypts of the adult rat has been measured by a stathmokinetic technique using vincristine. The value for the whole crypt column was 0·43 hr, or 26 min. At the bottom of the crypt t_m was in excess of 1 hr, but rapidly decreased and throughout the greater part of the proliferative compartment was between 0·40 and 0·50 hr. At the top of the proliferative compartment an increase in t_m was demonstrated.

If the value of 0·43 hr for the whole crypt column is correct, then one argument for postulating the formation of metabolic DNA during differentiation in the small bowel epithelium of the rat becomes invalid. Variations in t_m within the crypt have been shown to increase the values of cell velocity obtained from cumulative birth rate diagrams. Finally further evidence has been presented for the existence of a slowly dividing subpopulation of cells at the base of the crypt. These cells may be important in crypt repopulation after damage with phase specific anti-tumour drugs.

INTRODUCTION

The duration of mitosis in the crypts of the small intestine has recently emerged as an important parameter, and has attracted considerable attention. The major reason for this interest has been the claim by Pelc (Appleton, Pelc & Tarbit, 1969) that formation of metabolic DNA occurs in the small bowel epithelium of the mouse during differentiation. One basic argument upon which this claim depends is the discrepancy between the observed ratio of labelled to mitotic cells (the I_L/I_m ratio) and that derived by calculation from the known duration of DNA synthesis (t_s) and of mitosis (t_m). These ratios were found by Pelc to be 8·6 and 15·3 respectively. Central to this argument is t_m itself; Appleton *et al.* (1969) noted that a decrease of t_m from their assumed value of 0·80 hr to 0·45 hr was accompanied by a resolution of the difference existing between the observed and the calculated I_L/I_m ratios. The value of 0·80 hr for mouse small bowel epithelium was obtained from the work of Lesher (1967), who used the fraction of labelled mitoses (FLM) technique to measure t_m. In a later

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publication Pelc (1971) obtained a value of 1·04 hr for t_m in mouse small bowel using metaphase accumulation with colcemid. On the other hand, there is evidence for a different state of affairs in the rat small bowel epithelium. Clarke (1970), also using a stathmokinetic technique involving colcemid, has reported a value of 0·38 hr for t_m in rat small intestinal epithelium. If this latter value for t_m could be substantiated, then the formation of metabolic DNA need not be invoked. This matter is hardly academic. The calculation of mean transit times through the maturation compartment at the top of the crypt from the last division in the proliferative compartment (Cairnie, Lamerton & Steel, 1965a; Cleaver, 1967; Wright, Morley & Appleton, 1972) and, perhaps more important, the measurement of proliferative compartment size from crypt labelling index distribution diagrams (Cleaver, 1967; Wright & Morley, 1971) would both be affected by the incorporation of tritiated thymidine into DNA after the last division in the proliferative compartment.

The question may also be asked, what does a value of t_m obtained from the whole crypt population mean? It is evident from the work of Cairnie *et al.* (1965a) that variations in some cell cycle parameters exist between cells at different positions in the crypt. It is also possible that similar variations could occur in t_m. If this were the case, the value obtained for t_m may depend to some extent on the fraction of the crypt population studied, and it would be necessary to define accurately the population under consideration. From a practical viewpoint, a knowledge of variations in t_m within the crypt population is important in the construction of cumulative birth (or mitotic) rate curves. This technique (Cairnie *et al.*, 1965a, b; Wright *et al.*, 1972) allows assessment of cell velocity within the crypt population, and was used by Cairnie *et al.* (1965b) to calculate transit times at different cell positions within the crypt population in the substantiation of their slow cut-off model.

For these several reasons it was decided that an attempt should be made to measure t_m accurately in the rat small bowel, and to assess any variations which may occur in t_m with cell position in the crypt. Such an investigation might also be expected to cast some light on a subpopulation of cells at the base of the crypt which may have a long cell cycle time (T_c). Cairnie *et al.* (1965a) suspected that these cells had a T_c in excess of 50 hr. Lamerton (1972) has pointed out the possible significance of such a long cycling population in repopulation of the crypts after chemotherapeutic or irradiation damage.

MATERIALS AND METHODS

Male albino Wistar rats aged 3 months and weighing 150–200 g were used throughout. All injection procedures were carried out at 09.00 hours. This rigid timing was chosen in an attempt to avoid the effects of diurnal variation in I_m noted by Sigdestad, Banman & Lesher (1969). Animals received 1 mg of vincristine sulphate (Oncovin, Lilly) per kg body weight by intraperitoneal injection. The rats were killed by cervical dislocation. Samples of small bowel were taken from the region immediately below the ligament of Treitz, and thus would be described as upper jejunum. The tissue was fixed in Carnoy's fluid for 6 hr and paraffin embedded. Transverse sections were cut at a thickness of 3 μm and stained with Harris's haematoxylin.

Five normal, uninjected animals were killed at 09.00 hours. One vincristine treated animal was killed every 10 min up to 160 min after vincristine. A further four animals were also killed 150 min after vincristine. Lala (1971) has given equations for the calculation of t_m using two readings only, a native mitotic index at nought time, $I_{m(0)}$, and a mitotic index after time t, $I_{m(t)}$. As this method may be applicable to humans, where only two biopsies are feasible, this

method, using the readings at 0 and 150 min, was tried to compare it with the more accurate method of calculating mitotic duration in which mitotic rate is measured directly from the metaphase accumulation line.

In each animal 100 crypts were selected. Only complete axial crypt sections were chosen (i.e. those in which the base, middle and mouth of the crypt were all in the plane of section). Care was taken to ensure that each crypt was analysed only once. The counting techniques of Cairnie *et al.* (1965a) were used throughout. Slides were randomized before counting commenced. In each crypt the left-hand column of cells was numbered, counting from the bottom upwards to the crypt–villus junction. The positions of nuclei in mitosis were recorded. A mitosis situated midway between the right- and left-hand columns was counted as a half (Cairnie *et al.*, 1965a). In the five untreated control animals all stages of mitosis were counted. In the animal killed at 10 min after vincristine injection a few telophases were still apparent, but thereafter only prophases and metaphases were seen and therefore counted.

In any sample of small intestinal crypt columns, considerable variation is found in column height (Cairnie *et al.*, 1965a; Wright *et al.*, 1972). Cairnie & Bentley (1967) found differences in the distribution of labelled cells in short and long crypts of normal animals if the positions of cells were taken relative to the top or bottom of the crypt, and the same argument applies to the distribution of mitoses. For this reason we have analysed the data by a modified version of the method used by Cairnie & Bentley (1967). This method uses a large 'standard' crypt.

Data for each crypt was stored on punched cards. The data was subjected to an ALGOL program run on an IBM 360/67 computer which produced graphical output. Let s be the number of cells in the standard crypt. This was taken to be 1000 for the results given. The standard crypt is represented by an array C with s elements $1, 2, \ldots, s$. Originally each element of C is set to zero.

Consider an actual crypt of size m with a cell in position r to which a score k is assigned. For example k may be equal to 0, $\frac{1}{2}$, 1, $1\frac{1}{2}$ or 2. This extension is required as mitoses may be found midway between two crypt columns and more than one mitosis may be present opposite the same cell position. Elements C_i are then increased by an amount km for each of the s/m cells from $i = (s/m)(r-1)+1$ to $i = sr/m$. This partitions the s cells of the standard crypt into equal lengths of s/m cells. When s/m is non-integer the nearest integer is used.

This method ensures that the total amount added is the same, i.e. s for a mitotic cell in a crypt of any size. In Cairnie & Bentley's method there was a bias towards the distribution in small crypts which is avoided by the technique described.

Results are printed corresponding to a crypt of any size p. The value chosen for p is conveniently the mean crypt size if crypts of similar sizes are being compared, as in the present investigation. The mitotic index for cell j of a crypt of size p if there are n crypts altogether is

$$I_{m(j)} = \frac{100}{n.s} \sum C_i \qquad (1)$$

where i takes values from $(s/p)(j-1)+1$ to sj/p.

As Clarke (1970) has pointed out, it is important to state criteria for the identification of the various mitotic stages. The morphological criteria used were similar to those of Clarke (1970). Prophases were scored from the appearance of chromosomes as threads of chromatin until the disappearance of the nuclear membrane. Metaphase was concluded by the start of separation of the two chromatin masses, and anaphase lasted until the distinct separation of

the chromatin masses without extended chromosomes. Finally telophases were registered until the reappearance of the nuclear membrane.

Tannock (1967) has indicated that an important geometrical factor is involved in the measurement of crypt mitotic indices (I_m) from tissue sections. Nuclei in mitosis are found out of line with the interphase nuclei of the remainder of the crypt column, and are found to be nearer the crypt axis. This spatial arrangement would be expected to lead to an overestimate of I_m. Tannock therefore proposed a constant a/b (where a is the radial position of mitoses in the crypt, and b is the radius of the crypt lumen), by which the mitotic index should be multiplied in order to correct for this error. Sections cut tangentially to the bowel were examined, and the values for the ratio a/b obtained by measurement of 210 approximately circular crypt sections containing metaphases using a calibrated eyepiece graticule.

The increase in mitotic incidence was then plotted against time. Lines of best fit were obtained by the least squares method, and 95% confidence limits for the lines were calculated.

One other factor which must be considered when calculating T_c for cells in the lowest positions in the crypt is the number of Paneth cells found in each position. These cells may not be engaged in proliferation (see Discussion) and if this is the case should be excluded from such calculations. Three normal uninjected animals were used to measure the Paneth cell distribution. In each 100 axially sectioned crypts stained with haematoxylin and eosin were examined and the percentage of Paneth cells at each cell position in the crypt was calculated. The Paneth cells were easily recognized by their abundant eosinophilic cytoplasmic granules.

RESULTS

The whole crypt column

In Fig. 1 is shown the mitotic index as a function of the cell position in the crypt for the untreated control animals. The distribution is similar to that reported earlier by Cairnie *et al.* (1965a) and Wright *et al.* (1972). Low indices, around 3–4%, are found in the first three cell positions, and this increases to a maximum at about position 10. A steady decrease follows, and very low indices are reached by cell position 25. The fraction of the crypt occupied exclusively by the proliferative compartment is the region over which mitotic cells are observed, and this region was considered to end approximately at the cell position where the

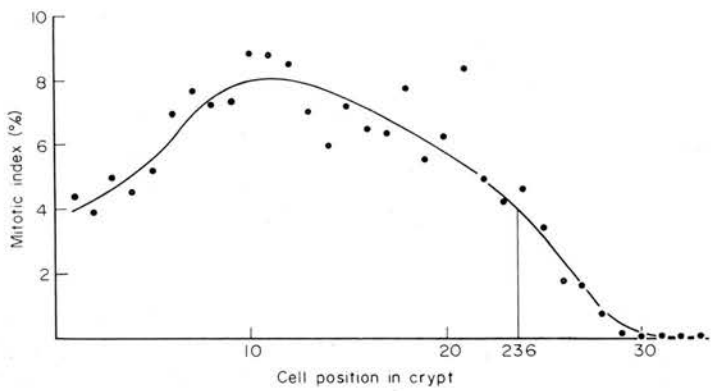

FIG. 1. The variation in mitotic index plotted as a function of cell position in the crypt. The perpendicular line is drawn at the cell position of the 50% peak or maximum value.

I_m fell to 50% of the maximum or peak value (Cleaver, 1967). This was found to be at cell position 24. The growth fraction can be defined as the ratio of the number of cells in the proliferative compartment to the total number of cells in the crypt column, i.e. 24/33 or 0·72. Calculation of the growth fraction from mitotic index distribution curves is very approximate, and would be expected to give higher values than those obtained from labelling index curves (see Discussion).

The increase in I_m with time after treatment with vincristine is shown for the whole crypt column in Fig. 2, and it can be seen that for the duration of the arrest period the graph shows a good linear relationship with $r^2 = 0{\cdot}94$. The slope of the graph is therefore a measure of the rate of entry into mitosis for the whole crypt column. This value was 11·16% of cells per hour.

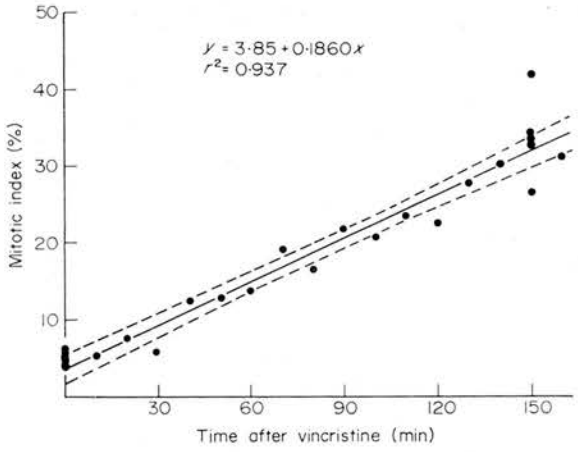

FIG. 2. The mitotic accumulation line after vincristine for the whole crypt column. The broken lines indicate 95% confidence limits for the line.

From the rate of entry into mitosis an estimate can be made of the cell cycle time, T_c. Tannock's constant a/b, obtained by the method described above, was 0·62 and compared well with Tannock's value of 0·57 for Marshall rats. Therefore R, the rate of entry of cells into mitosis per proliferating cell of the population, is related to the rate of entry as a percentage by

$$R = \frac{11{\cdot}16}{0{\cdot}72 \times 100} \times 0{\cdot}62 = 0{\cdot}097$$

for a growth fraction of 0·72.

Assuming steady state conditions within the population of the crypt column, the mean cell cycle time is the reciprocal of the rate of entry into mitosis or,

$$T_c = 1/R = 1/0{\cdot}097 = 10{\cdot}4 \text{ hr}$$

This value compares well with the T_c obtained by Cairnie *et al.* (1965a) for the whole crypt column of August rats of 10·5 hr by the FLM method. It should be noted that as the experiment occupied a finite time period, the measurement of R could be affected by diurnal variations in I_m (Sigdestad *et al.*, 1969). This was compensated to some extent by adhering to a standard vincristine injection time.

However, as noted above, the value used for the growth fraction is high. As measured from labelling experiments (Tannock, 1967) the growth fraction is 0·58. If this value is used T_c becomes 8·4 hr, which is shorter than the estimate of Cairnie *et al.* (1965a).

The duration of mitosis (t_m) for the whole crypt column can be found from the ratio of the native I_m ($I_{m(0)}$) to the rate of entry into mitosis (q) obtained from the slope of the accumulation graph, both expressed as a percentage

$$t_m = \frac{I_{m(0)}}{q} \tag{2}$$

This gives a value of 0·43 hr or 26 min. This value for t_m is similar to that obtained by Clarke (1970) but is much lower than the value of 1·04 hr given by Pelc (1971) in the mouse. The value obtained using Lala's (1971) formula and assuming linearity was similarly 0·43 hr.

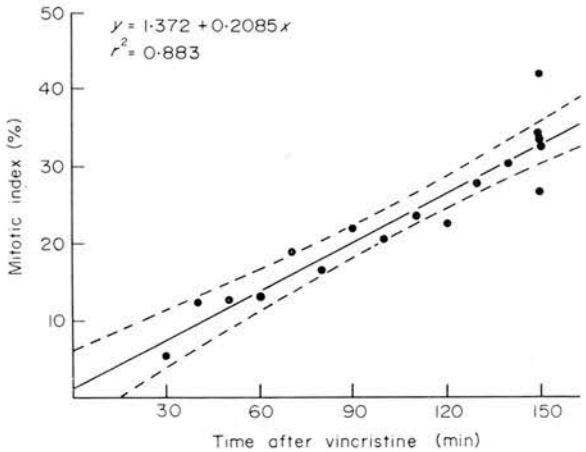

FIG. 3. The mitotic accumulation line after vincristine for the whole crypt column, but excluding the readings prior to 0·5 hr. The broken lines indicate 95% confidence limits for the line.

Clarke (1970) used metaphase accumulation from 0·5 to 2·5 hr after colcemid injection and similarly Pelc (1971) ignored the native I_m in the construction of the metaphase accumulation line. The rationale here is that events during the early period of action of the stathmokinetic agent are uncertain, and these procedures effectively remove the suspect period. However, Taylor (1965) has shown that *in vitro* the action of a stathmokinetic agent on cells already in mitosis may be related to its concentration, and similar considerations may apply *in vivo* (see Discussion). In the drawing of Fig. 2 vincristine has been assumed to become effective as soon as it is injected. The practical importance of this assumption can be gauged from Fig. 3 in which is plotted the metaphase accumulation graph with readings prior to 0·5 hr omitted. There is some difference in the lines; the confidence limits for the first set do not always include the estimate for the second set, and the second line (Fig. 3) fits the data less well than the first line (Fig. 2). This poorer fit may be related to the fewer observations. The rate of entry into mitosis is increased to 12·4% per hour. This value gives a T_c of 9·4 hr and a t_m of 0·39 hr (23 min) compared with the previous values of 10·4 hr and 0·43 hr. In practical terms, this method makes little difference (0·05 hr) to t_m, and leads to a reduction in T_c of about 1 hr.

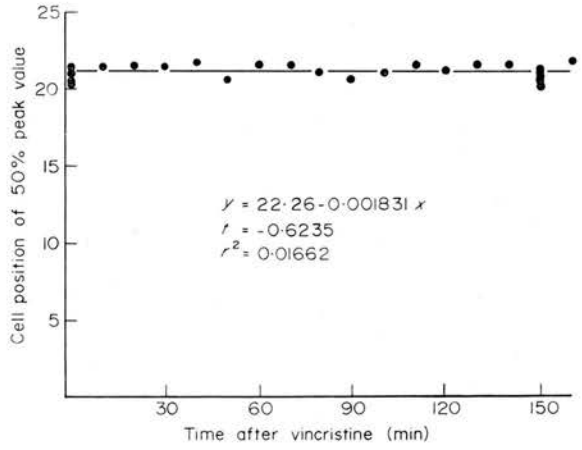

FIG. 4. Variation in the position of the 50% peak or maximum value with time, after vincristine. The 50% peak values for the five animals killed at 0 time, and at 2·5 hr, are given as single observations.

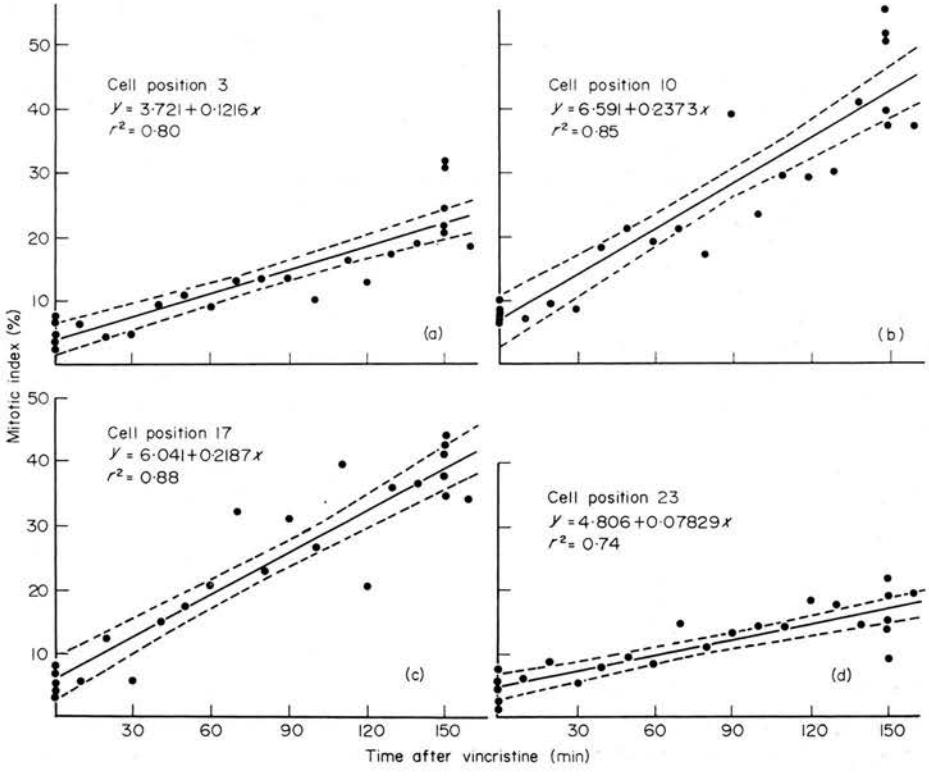

FIG. 5. Mitotic accumulation lines for four cell positions in the crypt column. The broken lines indicate 95% confidence limits for the lines.

A further method which can be used to calculate t_m from Fig. 2, again considering vincristine effective immediately, is to extrapolate the line backwards. The negative intercept on the x-axis is then t_m, and gives a value of 0·34 hr (21 min). Each of these methods describes the data equally well.

Variation in t_m with cell position

A basic assumption in the measurement of t_m at various positions using a stathmokinetic technique is that after metaphase blockade the arrested metaphases do not move with respect to the adjacent interphase nuclei. This assumption was checked by measuring the cell position of the 50% peak (or maximum) value on the mitotic distribution curve at each time interval. This method has been used with respect to labelled cells in the assessment of cell velocity by Cairnie *et al.* (1965a) and Wright *et al.* (1972). The movement of the 50% peak value with time is shown in Fig. 4, and from this it was concluded that no movement of arrested metaphases occurred throughout the duration of the experimental arrest period.

Mitotic accumulation graphs were constructed at each cell position and Fig. 5 shows representative results from various cell positions in the crypt. Up to cell position 24, r^2 values for the regression lines of 0·60 or above were obtained. Above position 24, however, r^2 values of below 0·50 were apparent.

The variation in the rate of entry of cells into mitosis is given in Fig. 6 together with 95% confidence limits of the values. Low rates are found at the base of the crypt in the first few positions, but this quickly rises to a maximum at position 10. The rate then falls to very low values after position 23, and even if those cell positions giving low r^2 values are excluded an apparent decrease in the rate of entry of cells into mitosis occurs as one moves towards the top of the crypt. These values are merely the slope of the regression lines and should be multiplied by 0·62 (Tannock's constant) for absolute values.

The variation in t_m calculated for assumed steady state conditions from equation (2) is shown in Fig. 7. Longer durations are apparent in the first three cell positions, approaching

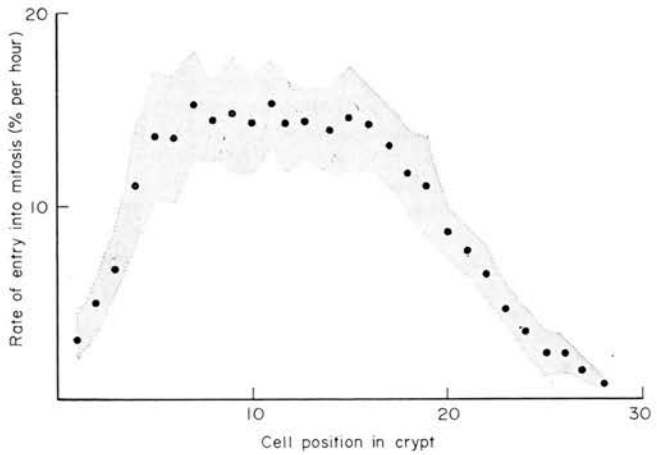

FIG. 6. The rate of entry into mitosis calculated from the slopes of the vincristine regression lines for each cell position in the crypt. The shaded area indicates 95% confidence limits for the values. For absolute values, Tannock's constant (0·62) should be taken into account.

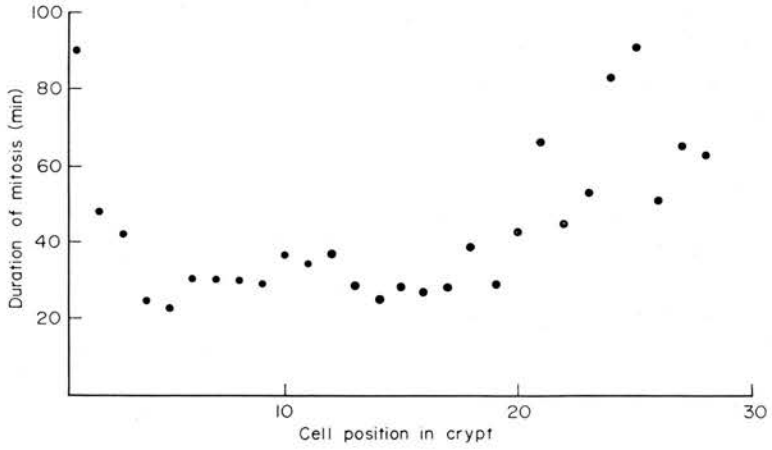

FIG. 7. The variation in mitotic duration with cell position in the crypt, calculated from the mitotic accumulation line assuming steady state conditions.

1 hr, but then in the remainder of the proliferative compartment values for t_m of between 20 and 30 min are obtained. At cell position 20 and thereafter t_m again increases in duration and values approaching 1 hr are found. Again, if the higher cell positions giving low values for r^2 are omitted, it is still apparent that a lengthening in the duration of t_m does occur towards the top of the proliferative compartment.

It is concluded from these observations that variation in t_m with cell position in the crypt does indeed occur, and such variations should be considered when single values of t_m are given for the whole crypt population, and in the calculation of parameters involving the use of the mitotic duration.

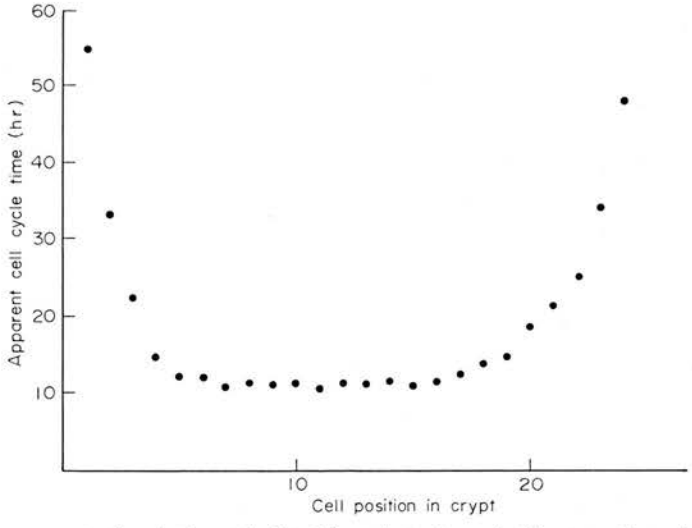

FIG. 8. The apparent cell cycle time calculated from the reciprocal of the rate of entry into mitosis, for each cell position in the crypt.

It is possible, using the present data, to provide some idea of proliferation rates occurring at the base of the crypt. Fig. 8 shows the apparent cell cycle time $T_{c(a)}$ for each cell position, calculated from the reciprocal of the rate of entry into mitosis as before with the assumption that all cells are in the cell cycle, i.e. the growth fraction at each cell position is 1. As would be expected from Fig. 6 showing variations in the rate of entry into mitosis, similar changes in $T_{c(a)}$ are found. From Fig. 8 it can be seen that the $T_{c(a)}$ from cell position 5 to 16 falls between 10 and 12 hr, which compares well with the estimates obtained for these cell positions by Cairnie *et al.* (1965a) by the FLM technique. In these cell positions it is reasonable to propose that the growth fraction is equal to 1 (Cleaver, 1967). It is apparent that a longer $T_{c(a)}$ is found in the first four cell positions, and $T_{c(a)}$ also lengthens towards the top of the proliferative compartment. If the cells at the base of the crypt constitute a 'stem cell compartment' for the crypt, it can perhaps be suggested that the growth fraction equals 1 (Lamerton, 1972). In this

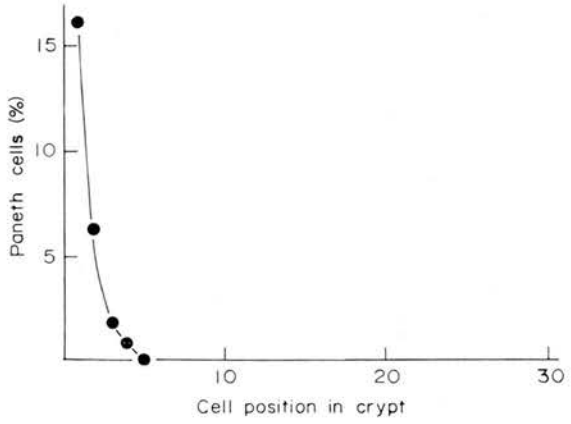

FIG. 9. The percentage incidence of Paneth cells in the crypt column of the rat jejunum.

case $T_{c(a)}$ can be equated with T_c and therefore cells in this region are moving slowly through the cell cycle. At the top of the proliferative compartment, however, cells are beginning differentiation and are moving out of the cell cycle. Consequently the growth fraction is falling and this may be responsible for the increased $T_{c(a)}$ values obtained. The percentages of Paneth cells for each cell position in the standardized crypt are shown in Fig. 9. At position 1, 16·0% of cells are Paneth cells, and in succeeding positions the values fall abruptly to reach 0·7% at cell position 4. This curve is similar to that obtained by Cairnie (1970) for August rats.

DISCUSSION

The value of 0·43 hr for t_m in the rat jejunum obtained in this investigation is comparable with that of Clarke (1970) who found 0·38 hr for the terminal ileum of the rat. Similar values for t_m using techniques involving disappearance of mitoses after irradiation have been given by Widner, Storer & Lushbaugh (1951). These workers obtained 0·42 hr, confirming the earlier value of 0·40 hr found by Knowlton & Widner (1960). These values are at variance with some earlier observations (Leblond & Stevens, 1948) and Clarke (1970) has proposed several

reasons for the longer times found by these workers. More recently Pelc (1971) using colcemid has published a value of 1·04 hr for t_m in the whole crypt of the mouse, and this extended value was given as evidence of the occurrence of metabolic DNA during differentiation in the small bowel. One reason for the observed differences in t_m between rat and mouse small intestinal epithelium may of course be a genuine species difference. If the present value for t_m is correct, then one reason for proposing the formation of metabolic DNA in the rat small bowel becomes redundant. It is also concluded that variation in t_m occurs with position in the crypt, and it therefore becomes essential to define accurately the limits of the population considered in the measurement of such a parameter.

A further context in which such variations become important is in the construction of cumulative birth or mitotic rate curves (Cairnie *et al.*, 1965a). The birth (or mitotic) rate is given by the ratio of the mitotic index to the mitotic duration, and the velocity of a cell at a particular position (and the efflux at that position) will be equal to the cumulative birth rate

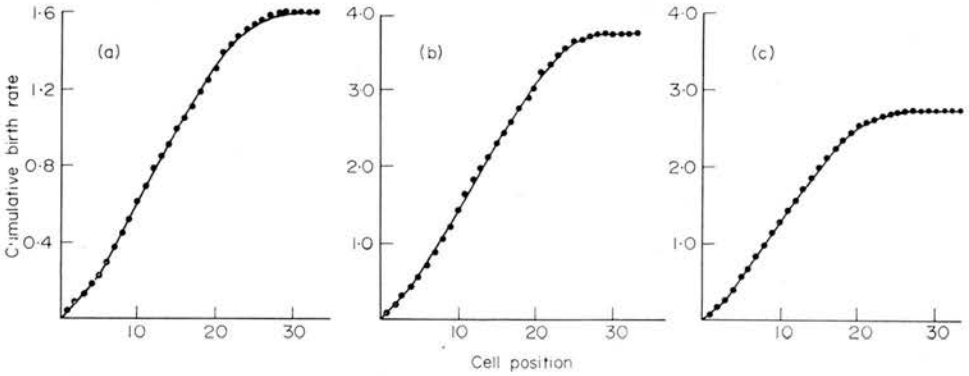

FIG. 10. Cumulative birth rate curves calculated, (a) assuming a constant t_m of 1·0 hr, (b) assuming a constant t_m of 0·43 hr, and (c) using the variable t_m of the present results.

of the cells up to that position. Cairnie *et al.* (1965a), using an assumed value for t_m of 1 hr, obtained a summating or limiting velocity at the top of the proliferation compartment of 1·45 cell positions/hr. Fig. 10 shows cumulative birth rate curves drawn for the present results using values of 1 hr and 0·43 hr for the whole crypt (Fig. 10a and b) and using the variable t_m described above (Fig. 10c). With 1 hr, a summated value of 1·6 cell positions per hour was found, but with the variable t_m this value rose to 2·6 cell positions per hour. One factor which has not been included in these calculations is Tannock's constant, 0·62. When this is included a summated value of 0·99 cell positions per hour is obtained when t_m is constant at 1 hr. When the variable t_m is used, this value becomes 1·6 cell positions per hour. It is evident that considerable errors occur when a constant value for t_m is assumed to obtain throughout the crypt, and a lower t_m would be expected to give a larger summating velocity.

The variations in rate of entry into mitosis with cell position depicted in Fig. 6 are complicated by similar variations which may occur in the growth fraction. Earlier it was noted that if the sub-population of cells with long apparent cell cycle times at the base of the crypt were stem cells and had a growth fraction of unity, then these values become real cell cycle times. A further complication is the presence in this region of Paneth cells, about the proliferative properties of which opinions are divided. Thrasher & Greulich (1966) considered that these

cells constituted a renewing population and were able to obtain a T_c of 80 hr by the FLM technique using stage-duration calculations. Similarly Deschner (1967) was able to demonstrate proliferation in human Paneth cells. Cairnie (1970) and Merzel & Leblond (1969), however, consider that Paneth cells are not normally engaged in the cell cycle. If the latter argument is true, then errors will appear in the measurement of rate of entry into mitosis in these positions. In Table 1, $T_{c(a)}$ for the lowest cell positions is given when Paneth cells are both included and excluded from the calculation. It can be seen that, even if Paneth cells are considered to be non-proliferative and are excluded, values for $T_{c(a)}$ are found which are considerably longer than the 10–11 hr obtained for the more rapidly dividing portion of the proliferative compartment.

TABLE 1. Apparent cell cycle times, $T_{c(a)}$, in the lowest cell positions, with and without Paneth cells included in the proliferative population

	Cell position			
	1	2	3	4
$T_{c(a)}$ all cells	55	33	22·2	14·7
$T_{c(a)}$ without Paneth cells	45	31	21·7	14·5

Cairnie *et al.* (1965b), in the formulation of their slow cut-off model, postulated that cells in the lower part of the crypt formed an exponentially growing population. In this case, $T_{c(a)}$ should be calculated, not from the reciprocal of the rate of entry into mitosis, but from $T_{c(a)} = \ln 2/R$. This would have the effect of further reducing $T_{c(a)}$ at the base of the crypt. In cell position 1 for instance, $T_{c(a)}$ becomes 31·5 hr, which is still prolonged, especially as $T_{c(a)}$ in the succeeding cell positions are reduced in proportion.

If, as is suggested, a subpopulation of cells with longer cell cycle times does exist then this could be a critical factor in crypt repopulation after chemotherapeutic insult with phase specific anti-tumour drugs (Lamerton, 1972) and could explain the relative sparing of the small bowel which occurs when experimental animals are treated with these agents (Skipper & Schabel, 1971). Cairnie *et al.* (1965a) estimated that the cell cycle time in cell position 1 might be as long at 50 hr. It is of obvious importance to investigate the possible role of these cells in crypt repopulation.

At the top of the crypt, the rate of entry into mitosis, and therefore the apparent cell cycle time, is seen to decrease. The probable cause of this decrease is a fall in growth fraction as cells leave the proliferative compartment and enter the maturation compartment. Cairnie *et al.* (1965a), using the FLM technique, found that T_c became shorter towards the top of the proliferative compartment; substantiation of this point by the present method would mean estimation of the growth fraction for each cell position, a most difficult procedure. It should perhaps be noted that unlike the rate of entry into mitosis, the mitotic duration calculated for steady state conditions (equation 2) is independent of growth fraction considerations. The latter parameter appears in numerator and denominator and therefore cancels out.

Vincristine was used in this investigation because Tannock (1967) obtained values for T_c using this agent which compared well with T_c obtained by the FLM technique. The dose used, 1 mg/kg body weight, is larger than that used by Tannock, but gave reproducible and reliable

metaphase arrest. As Taylor (1965) has demonstrated for colchicine *in vitro*, the concentration used determines whether immediate action upon mitotic cells occurs. Tannock (1967) did not demonstrate any lag in time before metaphase blocking with vincristine. However, this is not very important in measurement of t_m, as was demonstrated above. Tannock also found the limits of linearity for vincristine was about 2·5 hr, and certainly we have demonstrated a good degree of linearity over 160 min.

Another important point is the lack of movement of the 50% peak after vincristine metaphase blockade. This not only means that measurement of mitotic duration on a cell positional basis is reasonable, but also indicates that, within the terms of reference of the crypt column, cell movement within the crypt may depend solely upon crypt mitotic activity. It should be noted that the measurements of the 50% peak values from individual animals at time 0 (Fig. 4) are slightly lower than the value obtained when all animals are considered together (Fig. 1). This is because definition of the peak value when 500 crypts are used is substantially clearer than when only 100 are available (Wright *et al.*, 1972).

The counting of objects in tissue sections has obvious drawbacks (Clarke, 1967, 1970) and a further factor is the exaggeration of the mitotic index by the spatial distribution of mitoses within the crypt. This latter factor can be allowed for by the use of Tannock's constant. For example, the measured rate of entry into mitosis of 11·16% of cells per hour should be multiplied by 0·62, Tannock's constant, giving 7·02%. That this appears reasonable is shown by the fact that in 10 hr, the cell cycle time of the whole crypt, 10×7 or 70% of cells will have entered mitosis. A value of 0·72 was obtained for the growth fraction. The use of crypt squashes (Clarke, 1970) is ideal when data about the whole crypt is required, but when variation in parameters within the crypt are to be measured, then axially sectioned crypts must of necessity be studied.

The value obtained for the growth fraction from the mitotic distribution curve is higher than previous values. Cleaver (1967) calculated a growth fraction of 0·58 from the labelling index distribution curve for August rats obtained by Cairnie *et al.* (1965a). The value calculated from FLM measurements assuming steady state conditions was 0·57, clearly good agreement. The value derived from the mitotic index distribution curve will, of course, be higher because the mitotic index falls later than the labelling index as cells progress up the crypt, as would be expected on the basis of the slow cut off model. As a means of obtaining absolute growth fraction measurements this method is only approximate, but could be employed in instances where differences in growth fraction are sought.

When only two measurements are used, i.e. at time 0, $I_{m(0)}$, and 150 min, $I_{m(t)}$, and substituted in Lala's (1971) equation for steady state conditions

$$t_m = \frac{I_{m(0)}\, t}{(I_{m(t)} - I_{m(0)})} \tag{3}$$

a value of 0·43 hr is obtained, obviously comparing well with the previous measurement. This could indicate that measurements of t_m made on a similar time interval and using only two observations might be expected to give reasonable results when steady state conditions are assumed. Such considerations may be important in estimation of t_m in human subjects.

There have been few previous attempts to measure variation in t_m with cell position. Romanov & Rybakov (1970) were able to demonstrate a shorter t_m in the upper third of the crypts in young rats. These workers, however, used a collection period of 6 hr, and as Tannock (1967) has shown, this extended period is too long to expect linear collection of metaphases.

ACKNOWLEDGMENTS

This work was supported by the North of England Cancer Research Campaign.

We thank Professor A. G. Heppleston for continued encouragement and advice. Mr R. Richardson and Mr H. Elliott rendered skilled technical assistance, and Mrs M. Heslop typed the manuscript.

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David Appleton

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15

STUDIES ON THE MECHANISM OF DIURNAL VARIATION OF PROLIFERATIVE INDICES IN THE SMALL BOWEL MUCOSA OF THE RAT

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(*Received* 17 *November* 1975; *revision received* 23 *March* 1976)

ABSTRACT

In the rat small bowel mucosa significant variation was found in both the labelling and the mitotic indices with time of day. The zenith and the nadir of labelling and mitotic activity coincided at 15.00 and 02.00 hours respectively. Small changes were found in the 'cut-off' position, but this variation in proliferative compartment size was insufficient to account for the comparatively wider fluctuations in proliferative indices. Measurements of the rate of entry into mitosis, using metaphase arrest with vincristine at three widely separated times during the day, showed no significant change.

Changes in the growth fraction or in the birth rate as measured cannot account for diurnal variation in the proliferative activity of the small bowel mucosa. We propose a hypothesis which involves diurnal fluctuations in the transit times through G_1 and through G_2.

The influence of diurnal variation on measurements of proliferative rates has only recently been quantified (Burns & Scheving, 1975). In respect of renewing cell populations such as that of the small bowel mucosa, few workers have assessed such data as cell cycle phase durations derived from FLM curves in the light of possible diurnal variation in proliferative rates or in phase durations. Despite early claims to the contrary (Pilgrim, Erb & Maurer, 1963), the detailed work of Sigdestad and Lesher (Sigdestad, Bauman & Lesher, 1969; Sigdestad & Lesher, 1970, 1971, 1972) has established that a definite diurnal rhythm of proliferative activity occurs in the small bowel mucosa of the mouse.

Brown & Berry (1968) demonstrated diurnal variation of proliferative indices in the cheek pouch epithelium of the hamster and sought to explain their data by postulating a partial synchronization of cells in the S phase. This produced a peak of labelling followed shortly by a peak of mitosis as cells completed G_2 and entered mitosis. On the other hand, in the small bowel Sigdestad & Lesher (1970) found coincident peaks in the labelling and

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mitotic index, in which case a partial synchronization in any one phase could not be responsible.

This paper reports an attempt to explain, in kinetic terms, the mechanisms underlying diurnal variation in proliferative activity in the small bowel mucosa of the rat. Knowledge of these mechanisms might well suggest how diurnal variation can affect measurements of cell cycle time.

MATERIALS AND METHODS

Male Wistar rats aged 3 months were used throughout. Animals were kept for 14 days in a constant temperature environment (18°C) with a dark/light schedule of 12 hr; the light phase was from 07.00 to 19.00 hours.

Experiment 1

Starting at 09.00 hours, three animals were killed every 2 hr over a 24 hr period. Animals were killed by cervical dislocation, and each received tritiated thymidine (^{3}H-TdR, Radiochemical Centre, Amersham), in a dosage of 0·5 μCi/g body weight, given by intraperitoneal injection 1 hr before death. Samples of upper jejunum were removed immediately distal to the ligament of Treitz, and were fixed in Carnoy's fluid for 6 hr. The material was embedded in paraffin, sectioned transversely at 3 μm, and autoradiographs were then prepared as previously described (Al-Dewachi *et al.*, 1974).

Experiment 2

Three groups of animals were injected intraperitoneally with vincristine sulphate (Oncovin, Lilly) at 09.00 hours (seventeen rats), 17.00 hours (eleven rats) and 01.00 hours (eleven rats). The dosage used was 1 mg/kg body weight. The animals were killed at intervals thereafter until 2·5 hr after vincristine injection. Samples of upper jejunum were removed, and 3 μm transverse sections were stained with haematoxylin and eosin.

In each animal 100 axially sectioned crypts were selected, and the distributions of labelled and mitotic nuclei in the left-hand crypt column were measured as previously described (Wright, Morley & Appleton, 1972a; Wright *et al.*, 1975). The method used to compensate for differences in the distribution of labelled and mitotic nuclei in crypts of different length is described by Wright *et al.* (1972a). Labelling index distribution curves were constructed.

The cell position at which the I_s falls to 0·5 of the peak value is useful as a reference point on the curve, and if the number of cell positions below this point is compared to the total number of cells in the standardized crypt column, a value for the growth fraction of the crypt column is obtained which compares well with values calculated from FLM curves (Wright, Morley & Appleton, 1972b; Wright *et al.*, 1975; Al-Dewachi *et al.*, 1975). Even if this ratio is not regarded as being synonymous with a crypt growth fraction, it represents a useful index of the cut off position (Cairnie, Lamerton & Steel, 1965a), which effectively controls the size of the proliferative compartment (Wright *et al.*, 1972b).

RESULTS

Diurnal variation in proliferative indices

Fig. 1 shows the labelling index (I_s) for the whole crypt column plotted against time of day. The dark component of the schedule is indicated by the black bar. From a value of

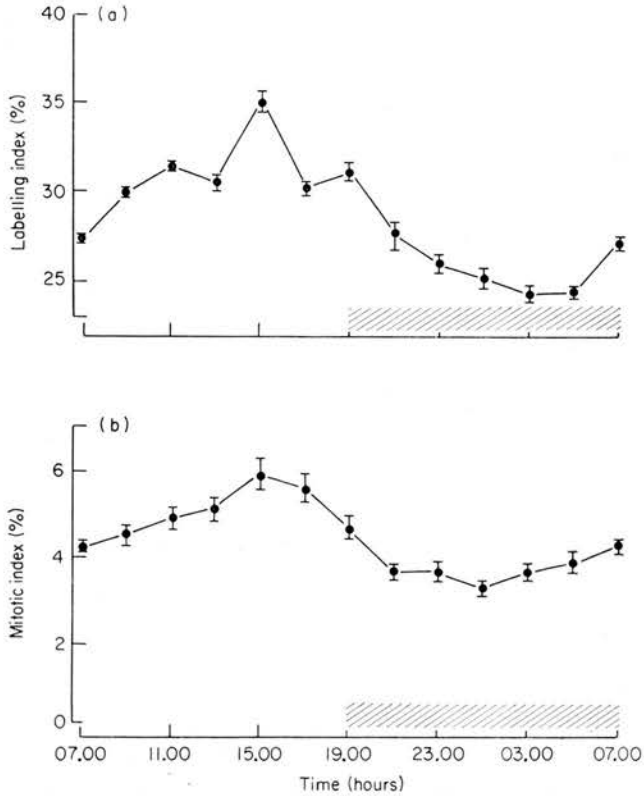

FIG. 1. The labelling index (a) and the mitotic index (b), both ± SE, measured at different times of day in the rat small bowel mucosa. The black bar indicates the dark phase of the schedule. Each point is the mean of three animals.

about 0·27 at 07.00 hours, I_s rises to reach a peak of 0·35 at 15.00 hours, within the light phase of the schedule. At 19.00 hours, the commencement of the dark phase, I_s has fallen to 0·32, and then falls to reach a nadir of about 0·24 at between 03.00 and 05.00 hours. That there is a real variation and not just random fluctuation may be shown by inspecting the number of successive observations above or below the median observation ($P < 0{\cdot}01$).

The changes in mitotic index (I_m) with time of day is also shown in Fig. 1. From a value of 0·042 at 07.00 hours there is a steady rise which reaches a peak of 0·059 at 15.00 hours. This is followed by a steady fall in I_m, realizing a nadir of 0·033 at 01.00 hours. I_m values then remain below 0·04 until 07.00 hours. Again the length of runs entirely above or below the median indicate a non-random pattern ($P < 0{\cdot}01$).

Fig. 2 shows I_s and I_m values plotted together. The zeniths of the two indices appear to coincide at 15.00 hours. To analyse these curves it becomes necessary to find out if they are in phase.

Since the variation in indices is periodic, it is useful to assume that they follow a form which can be approximated by a sine wave of period 24 hr; the data can then be fitted by the equation

$$x = \alpha + (\beta - \alpha) \cos \frac{\pi}{12} (t - \delta) \qquad (1)$$

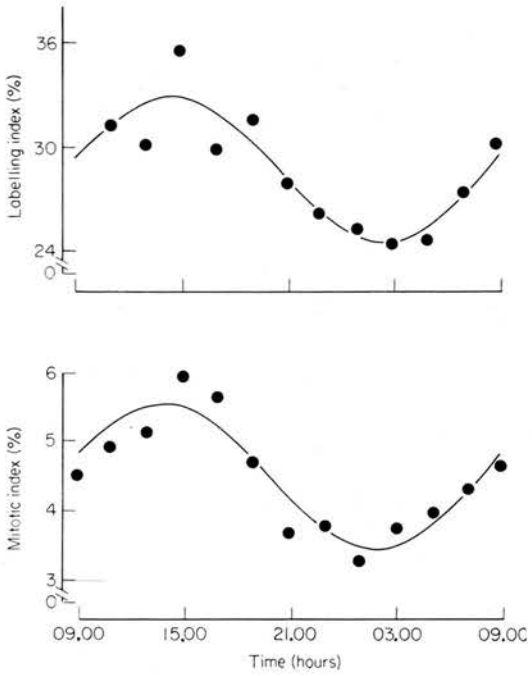

FIG. 2. The labelling index compared with the mitotic index. The lines are fitted by the method described in the text.

where x is the index and t the time in hours. Then α is the mean index, β the maximum and δ the time of the maximum. Fitting the curve by the method of maximum likelihood gives α, β and δ with standard errors; the values of δ from the labelling and mitotic index curves can be used to test for coincidence of peaks.

The curves in Fig. 2 are fitted using equation (1). A t value for coincidence of peak times is given by

$$t_\infty = \frac{\delta_{\text{labelling}} - \delta_{\text{mitotic}}}{\sqrt{[(\text{SE}\,\delta_{\text{labelling}})^2 + (\text{SE}\,\delta_{\text{mitotic}})^2]}}$$

$$= \frac{2\text{ hr }31\text{ min} - 1\text{ hr }59\text{ min}}{\sqrt{(34^2 + 31^2)}} = 0{\cdot}70$$

which is not significant. The power of the test is such that differences in phase of 2 hr 45 min would have a 95% probability of being detected at the 5% level.

It is concluded that a significant diurnal rhythm in I_s and I_m occurs, and that these are not significantly out of phase.

Changes in the distribution of labelled cells in the crypt column

In each animal the distribution of labelled cells was plotted against cell position in the crypt column, and some representative labelling index distribution curves are shown in Fig. 3. The formats of these curves are similar to those described hitherto (Wright *et al.*, 1975). Comparatively low indices are apparent in the lower cell positions, rising to maximum

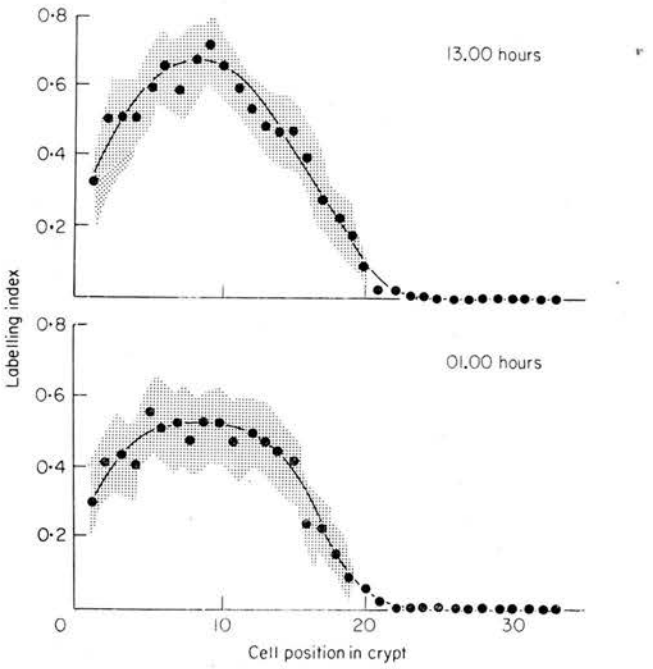

FIG. 3. A selection of representative labelling index distribution curves at different times throughout the day. The shaded areas indicate 95% confidence limits for the points.

values in the lower third of the crypt; at this point, within the proliferative compartment the experimental I_s attained equals the theoretical I_s as calculated from the duration of cell cycle phases for these cells (Cleaver, 1967; Wright *et al.*, 1975), and consequently the growth fraction in this region of the crypt approaches unity. Thereafter I_s falls towards the top of the crypt as cells decycle according to the slow cut-off model (Cairnie, Lamerton & Steel, 1965b; Maurer, Burholt & Schultze, 1975).

The values of the 'growth fraction' are plotted against time of day in Fig. 4. Between 09.00 and 21.00 hours values are relatively constant at around 0·51. After 21.00 hours a

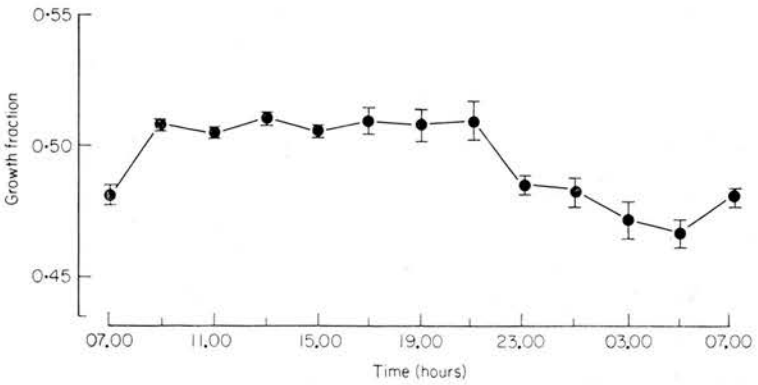

FIG. 4. Changes in the growth fraction (see text) as measured from the labelling index distribution curve at different times throughout the day.

steady fall is apparent reaching a nadir of about 0·46 at 05.00 hours. The length of runs entirely above or below the median indicate a non-random pattern ($P < 0{\cdot}01$). The extent to which this change in the growth fraction could be responsible for the demonstrated variation in proliferative indices is discussed below.

Metaphase arrest with vincristine

The mitotic accumulation lines after vincristine injection are shown for the times 09.00, 17.00 and 01.00 hours in Fig. 5. Because of uncertainties concerning the onset of metaphase arrest during the early period of vincristine action (Al-Dewachi *et al.*, 1975), the readings were begun at 30 min after injection. The line is fitted, assuming exponential conditions, using the equation

$$\log_2 (1 + I_m) = \frac{1}{T_c} (t_m + t) \qquad (2)$$

and the method of least squares.

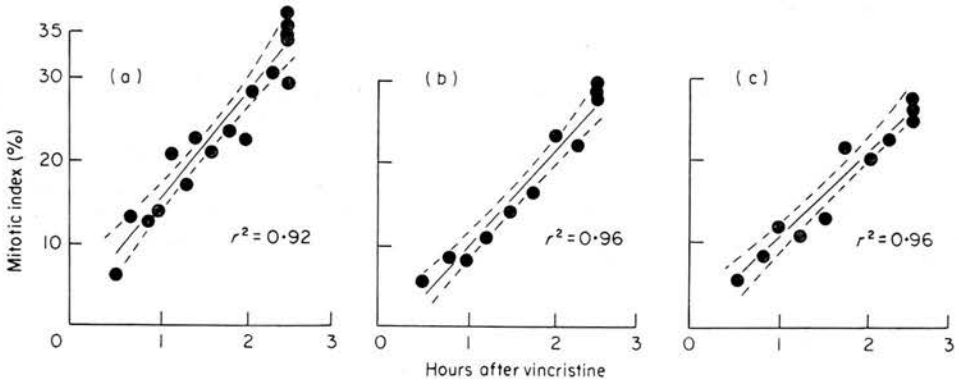

FIG. 5. Mitotic accumulation graphs after vincristine at (a) 09.00, (b) 17.00 and (c) 01.00 hours. The dotted lines indicate 95% confidence limits for the line.

The mitotic accumulation lines show a good degree of linearity over the experimental period. The birth rates, or rates of entry into mitosis, can be measured from the slope of the mitotic accumulation line. These values (with 95% confidence limits) were 14·8% (12·0, 17·6), 14·5% (12·5, 16·6) and 13·3% (11·2, 15·3) of cells per hour at 09.00, 17.00 and 01.00 hours respectively, and are not significantly different. A value for the apparent cell cycle time $T_{c(a)}$ can be derived from the reciprocal of this birth rate value, incorporating Tannock's constant (Tannock, 1967; Wright *et al.*, 1972a); $T_{c(a)}$ values (with 95% confidence limits) were 10·9 hr (9·2, 13·4), 11·1 hr (9·7, 12·9) and 12·2 hr (10·5, 14·4) at 09.00, 17.00 and 01.00 hours respectively (see Table 1). The birth rate obtained at 01.00 hours, at which time the mitotic index had reached its nadir, was the lowest value obtained, and gave the longest $T_{c(a)}$ value. It is improbable that this is the cause of the low proliferative indices at this time, since the birth rate at 01.00 hours does not differ significantly from the birth rate at 15.00 hours, which is close to the peak of proliferative activity. In addition, the highest birth rate attained was at 09.00 hours, at which time the proliferative indices are beginning the rise to their maxima at 15.00 hours. Evidently the stathmokinetic method does not allow sufficient discrimination for the detection of differences in birth rates, even

TABLE 1. Diurnal variation in some kinetic parameters in the rat small bowel

	09.00 hours	17.00 hours	01.00 hours
Rate of entry into mitosis* (% per hr, with 95% confidence limits)	14·8 (17·6, 12·0)	14·5 (16·6, 12·5)	13·3 (15·3, 11·2)
$T_{c(a)}$† (hr, with 95% confidence limits)	10·9 (13·4, 9·2)	11·1 (12·9, 9·7)	12·2 (10·5, 14·4)
Migration rate‡ (cell positions/hr)	1·8	2·2	2·0
Crypt cell production rate§ (cells/crypt/hr)	39·7	48·4	44·6

* As measured by metaphase arrest with vincristine.
† Apparent cell cycle time.
‡ Calculated from a cumulative birth rate curve (Wright *et al.*, 1975).
§ Calculated from the product of the migration rate and the column count (Wright *et al.*, 1975).

though studies of proliferative indices indicate significant diurnal fluctuations in proliferative activity.

Calculations made of the migration rate (Table 1) from the cumulative birth rate curve (Wright *et al.*, 1975) gave similar values at the times measured, while calculation of the crypt cell production rate from the method described by Wright *et al.* (1975) gave values which, although showing a wider range, are not considered to be different (Table 1).

DISCUSSION

It may be concluded from this study that significant diurnal fluctuations in proliferative activity do occur in the rat small bowel mucosa, as indicated by the changes in labelling and mitotic indices. The timing of these changes is interesting, as the zenith and nadir are almost diametrically opposite to those described by Sigdestad & Lesher (1970), who described a peak of proliferative activity at 02.00 hours in the mouse, with the nadir at 15.00 hours. However, the studies of Scheving, Burns & Pauly (1972) showed changes in the small bowel mucosa of the rat with timings very similar to the present study.

This work confirms the observation of Sigdestad and Lesher that the peaks of labelling and mitotic indices are coincidental, and consequently the explanation of Brown & Berry (1968), who invoked partial synchrony of cells in S, cannot be applied to this situation. Because the zeniths coincide, any factors controlling the diurnal variability must be affecting cells in G_1 and G_2 at the same time.

Any attempt to analyse the mechanisms of diurnal variation is handicapped by the temporal basis of most cytokinetic techniques; measurement of cell cycle time and growth fraction by the FLM method in the rat small bowel would take at least 13 hr for a single reading. Consequently the shorter stathmokinetic technique would appear *a priori* to be the more promising. The finding of an upward movement of the cut-off position which coincides approximately with the demonstrated increase in labelling and in mitotic indices might suggest that growth fraction changes contribute to the changes in proliferative

activity. If we assume that the ratio depicted in Fig. 4 is equivalent to the growth fraction, we can simply calculate the effect of such a growth fraction change on I_s and I_m. It must be conceded that this growth fraction change would have only a very minor effect on the proliferative indices, for example raising I_s from its basal value of 0·25 to 0·28, which is very much smaller than the demonstrated changes in I_s depicted in Fig. 1. Furthermore, comparison of Figs. 1 and 4 shows that the fall in I_s and I_m is well established before any detectable fall in the cut-off position.

We were unable to detect any change in the birth rate at the three time periods at which it was measured. Assuming the changes in growth fraction shown in Fig. 5 are real, it can be calculated that the changes are too small to be detected using stathmokinetic techniques. Thus we cannot say that the fluctuations in I_s and I_m are the result of changes in the rate of entry into mitosis. This conclusion is at variance with those of Tutton (1973), who was able to demonstrate significant changes in the rate of entry into mitosis and in $T_{c(a)}$ values in the small bowel mucosa of Sprague-Dawley rats at different times of day, although only the basal crypt region was considered.

We have chosen to use an exponential age distribution in the analysis of the stathmokinetic experiments since we are only looking here at mitotic cells, which by definition are proliferative; the age distribution of the proliferating crypt cells is most probably exponential.

Thus we have confirmed that diurnal fluctuations in proliferative activity do occur, but we are unable to make positive statements concerning the mechanism. This may be due to the limitations of the techniques which, because of the short time scale of the changes, have to be used. Diurnal fluctuations in proliferative indices do not appear to be due mainly to changes in the size of the proliferative compartment mediated by movement of the cut-off position; since in the proliferative compartment most (if not all) cells are in cycle, recruitment from a G_0 reserve in this portion of the crypt is unlikely.

How then can this pattern of diurnal variation be explained? Any mechanism producing coincidental peaks of labelling and mitotic activity should be acting in both G_1 and G_2; if the probability of entering DNA synthesis and mitosis varied with time of day, then there would be diurnal variation in the transit times through G_1 and G_2; coincident peaks could be produced by this mechanism. It must be conceded, however, that the changes in the rate of entry into mitosis are insufficient to explain the diurnal swings of the mitotic index. The feasibility of this hypothesis could be checked by use of a model similar to that proposed by Brockwell (1975).

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Virchows Arch. B Cell Path. 31, 37–44 (1979)

Virchows Archiv B
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Variation in the Cell Cycle Time in the Crypts of Lieberkühn of the Mouse

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Summary. The durations of the phases of the cell cycle were measured at different levels in the jejunal crypts of male Balb/c mice. A mean cell cycle time of 12.3 h was found for the whole crypt. In cell positions 1 and 2, the cell cycle time was 16.7 h, and this time steadily decreased to a value of between 10 and 11 h for cell positions above 11. It is concluded that basally situated crypt cells in the mouse are cycling relatively slowly, and that they form the functional stem cell pool for the crypt. These cells may also compose the potential stem cell pool which repopulates the crypt after death of proliferative cells.

Key words. Mouse – Jejunum – Stem cell cycles.

Introduction

There has been considerable recent interest in the identity of the 'stem' cells, or 'clonogenic' cells in the intestinal crypt, and much work has been carried out using the mouse as an experimental model. There are essentially two conflicting viewpoints on the number and proliferative status of clonogenic cells in the mouse small intestinal crypt. Some workers (Hendry and Potten, 1974; Potten and Hendry, 1975) have proposed that only about half of the proliferative crypt cells are clonogenic and that the basal crypt cells, which they assume to be cycling slowly, are among these. Others (Withers and Elkind, 1970; Hagemann et al., 1971) take the view that *all* proliferative crypt cells are clonogenic, and studies based on the sensitivity of clonogenic crypt cells to tritiated thymidine and cytosine arabinoside, indicate that most of these cells are cycling quickly (Boarder and Blackett, 1976).

In normal circumstates the basal crypt cells can be regarded as functional stem cells in the sense that they continually replenish the cells in the proliferative compartment (Cheng and Leblond, 1974). We have previously shown (Al-Dewachi

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0340-6075/79/0031/0031/$01.60

et al., 1977) that in the *rat* the basal crypt cells are first to proliferate in response to death of proliferative cells resulting from administration of hydroxyurea; in these abnormal circumstances it appears that basal cells are repopulating the depleted crypt. In further *rat* studies using a variety of techniques, viz. the fraction of labelled mitoses (FLM) method (Cairnie et al., 1965; Al-Dewachi et al., 1974), stathmokinetic methods (Wright et al., 1972) and the continuous labelling method (Wright, 1978) it has been shown that basal crypt cells are cycling more slowly than cells in the proliferative compartment. We ourselves, (Al-Dewachi et al., 1975) using a metaphase arrest method, have shown how the apparent cell cycle time varies with cell position in the crypts of the *mouse* jejunum, but these findings may reflect changes in growth fraction rather than changes in cell cycle time.

Here we report direct measurements of the cell cycle time in the mouse jejunal crypt, using the fraction of labelled mitoses method, and our results show that basal cells do indeed tend to have extended cycle times compared with those higher up the crypt.

Materials and Methods

Male Balb/c mice aged 3 months were used throughout. All injection procedures were carried out at 09.00 h. Forty-five animals were given tritiated thymidine (^{3}HTdR, Radiochemical Centre, Amersham, England, of specific activity 5 Ci/mM), by intraperitoneal injection, in a dosage of 1 μCi per g body weight. For the first 17 h after ^{3}HTdR injection an animal was killed by cervical dislocation every 30 min, and thereafter one each hour up to 28 h.

Portions of upper jejunum were excised from the region immediately distal to the ligament of Treitz, fixed unopened in Carnoy's fluid for 6 h, and embedded in paraffin wax. Serial transverse sections were cut at a thickness of 3 μm, and autoradiographs were prepared using Kodak D19 and then fixed, washed, and stained through the emulsion with Harris's haematoxylin.

Axially sectioned crypts were selected for analysis, i.e., those crypts sectioned so that the base, middle and top of the crypt were all in the plane of section. The "left hand" column of cells (the crypt column) was divided into nine portions consisting respectively of cell positions 1 and 2, 3 and 4, ..., 15 and 16, and cell positions 17 and above. The mean crypt length was 26 cells. The mitotic index varies with cell position, maximum values appearing between cell positions 5 and 14 (Al-Dewachi et al., 1975). Mitotic indices in cell positions 1 and 2 and 17 and above are low; in positions 1 and 2 this is partly because of the large proportion of Paneth cells and in position 17 and above because of the increasing proportion of cells which have left the cell cycle. Crpyts were inspected until at least 20 mitoses had been seen in cell positions 1 and 2 and in positions 17 and above. Mitoses were of course more frequent in the intermediate cell positions, where at least 100 were counted in each group. In order to avoid the difficulty of recognising prophases and telophases in section only metaphases and anaphases were scored. The fraction of labelled mitoses was then plotted against time after injection of ^{3}HTdR, and the data were analysed by the method of Gilbert (1972). The method generates mean values for the time parameters of the cell cycle, with approximate standard errors, and the coefficients of variation (CV) of these times.

Results

Figure 1 shows the FLM curves for the whole crypt, and also for the different cell position groups. In each case the line is fitted to the data points by the

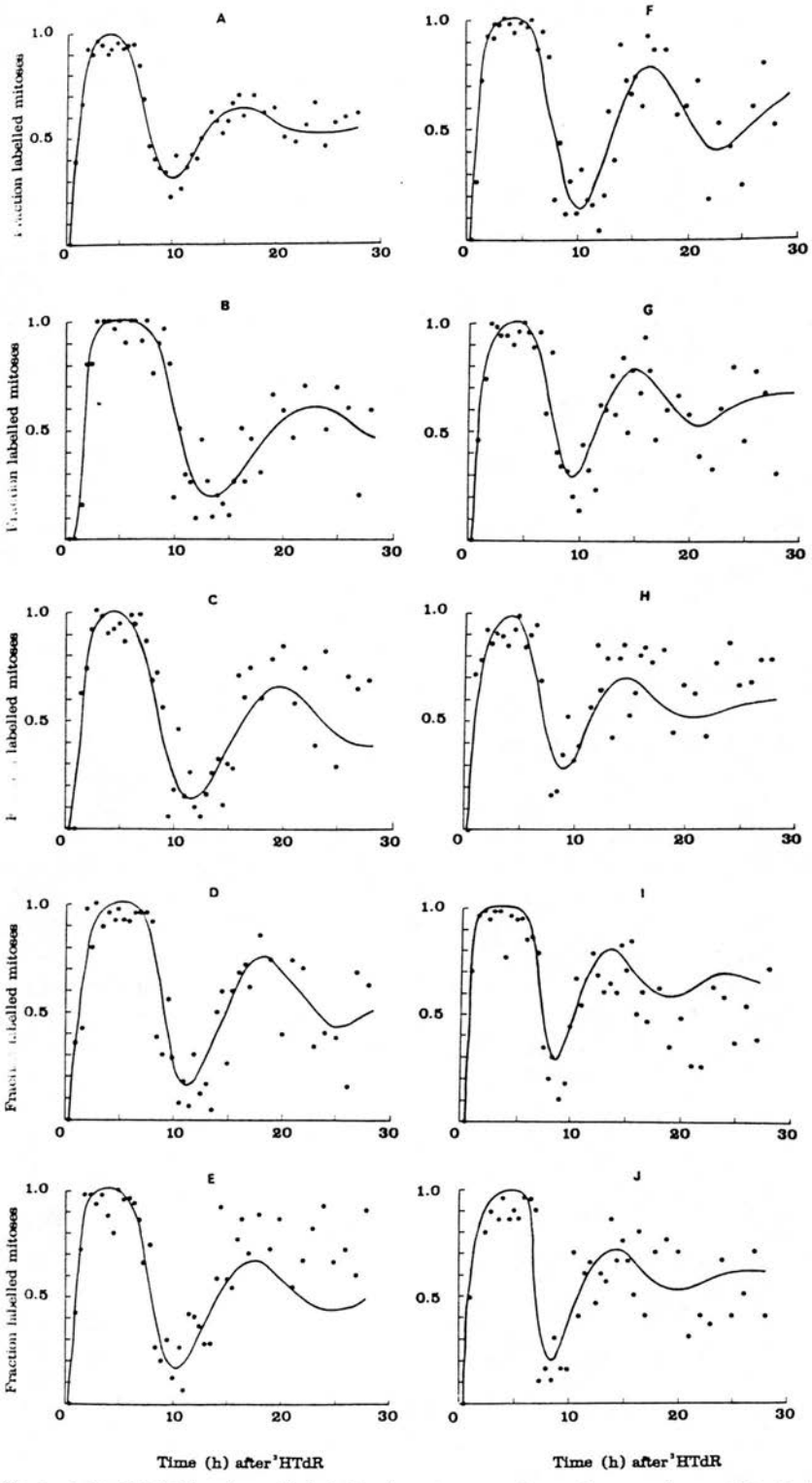

Fig. 1. A–J. FLM (Fraction of labelled mitoses) curves for various portions of the Balb/c mouse jejunal crypts. The experimental points are shown by the solid points, and the lines are fitted by the Gilbert program. **A** is the curve for the whole crypt, **B** is for cell positions 1 and 2, **C** for positions 3 and 4, and so on; **J** is for positions 17+

Table 1. Cell cycle parameters at different cell positions in the jejunal crypts of Balb/c mice

Cell position	Cell cycle time, T_c (h) mean ± standard error[a]	Coefficient of Variation of T_c (%)	t_{G_1}[b] (h)	t_S (h)	t_{G_2}[b] (h)
1–2	16.7 ± 0.3	29	6.2	8.4	1.6
3–4	14.9 ± 0.3	24	5.7	7.2	1.3
5–6	13.6 ± 0.2	22	4.3	7.5	1.2
7–8	13.3 ± 0.3	25	5.0	6.7	1.0
9–10	12.4 ± 0.1	19	4.0	6.7	1.0
11–12	10.9 ± 0.2	23	2.7	6.7	0.9
13–14	10.6 ± 0.3	26	3.1	6.0	0.9
15–16	9.7 ± 0.2	25	2.2	6.4	0.6
17+	10.3 ± 0.3	28	2.9	6.0	0.9
Whole crypt	12.3 ± 0.2	30	3.9	6.7	1.1

[a] Standard errors as given by Gilbert's method

[b] t_{G_1} and t_{G_2} have been reduced by 20 min each from the estimates given by the program to take into account the duration of mitosis

Gilbert program, and the fit is generally good. In all cases the fitted curve closely follows the first peak; in some instances, however, the curve fits the second peak less well. For cell positions 7 and 8 the fitted curve lies below the experimental points on the second peak, while for positions 15 and 16 it lies above. Possible reasons for these discrepancies are given in the discussion.

The mean values for the duration of the phases of the cell cycle, and the coefficient of variation for the cell cycle time (T_c), are given in Table 1. In the Gilbert program mitosis is taken to be an instantaneous event, and this of course affects the computed values of the G_1 period (t_{G_1}) and that of G_2 (t_{G_2}); the tabulated values have therefore been shortened by 20 minutes each. For the whole crypt T_c is 12.3 h ± 0.2 h (SE) and the duration of DNA synthesis (t_s) is 6.7 h. At the bottom of the crypt, the cell cycle time in cell positions 1 and 2 ($T_{c(1\text{ and }2)}$) is 16.7 h ± 0.3 h; the cell cycle time then becomes progressively shorter at succeeding cell positions. After cell position 11 the mean T_c remains essentially constant at about 10–11 hours.

Discussion

We do not wish to involve ourselves in the question of whether mean cell cycle times are significantly different at different cell positions, but following the same criteria we used previously in the rat (Al-Dewachi et al., 1974) it is reasonable to say that the mean cell cycle time up to position 8, at least, is significantly greater than that for cells above position 11. It is perhaps more informative to look at the distribution of cell cycle times than at the means.

The coefficient of variation of T_c varies between 19 and 29%: and, while there is no tendency for it to be larger in basal cell positions, the greater mean value there results in the variance of cell cycle times also being greater,

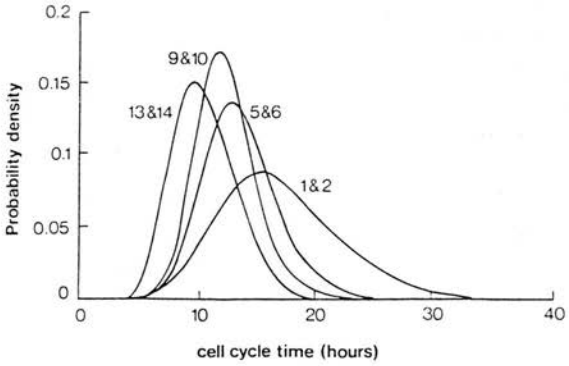

Fig. 2. Distribution of cell cycle times for various portions of the Balb/c mouse jejunal crypts, derived from the Gilbert program. (Numbers beside curves refer to cell positions)

Table 2. Percentages of cells with cell cycle time less than given values

Cell position	% cells with $T_c < 10$ h	% cells with $T_c < 12.3$ h	% cells with $T_c < 15$ h
1–2	6	18	40
3–4	7	24	54
5–6	11	35	70
7–8	16	41	72
9–10	16	51	86
11–12	39	73	94
13–14	45	75	93
15–16	58	86	97
17+	49	77	93
Whole crypt[a]	29	54	78
Whole crypt[b]	27	55	80

[a] Derived from the estimates of the mean and coefficient of variation of T_c for the whole crypt calculated by the Gilbert program

[b] Derived by weighting the percentage at each cell position by $T_c \times$ mitotic index (to take into account the changing growth fraction). Mitotic indices are from Al-Dewachi et al. (1975)

as in the rat (Al-Dewachi et al., 1974). Figure 2 shows the distribution of cell cycle times for four of the cell position groups, and Table 2 gives the approximate proportions of cells at each position which have cell cycle times below particular values. Ten hours was chosen as it is near the lowest value for basal cells, 12.3 h is the mean for the crypt, and 15 h is close to the longest cell cycle time for cells in the proliferative compartment.

The value obtained for the mean cell cycle time of the crypt as a whole (12.3 h) agrees well with previously published values for adult mice measured by the FLM method. Al-Dewachi et al. (1975) in a previous communication obtained 12.4 h, though with a coefficient of variation of only 21%; Thrasher and Greulich (1965) reported a value of 13 h, and Schultze et al. (1972) found a value of 14 h. No previous estimates are available of cell cycle parameters for different cell positions in the mouse crypt.

It is interesting to compare the present results, however, with results obtained from the same strain of mice using a stathmokinetic technique with vincristine (Al-Dewachi et al., 1975). This latter method gives values for the apparent cell cycle time ($T_{c(a)}$) since it includes non-cycling cells in the analysis, and when

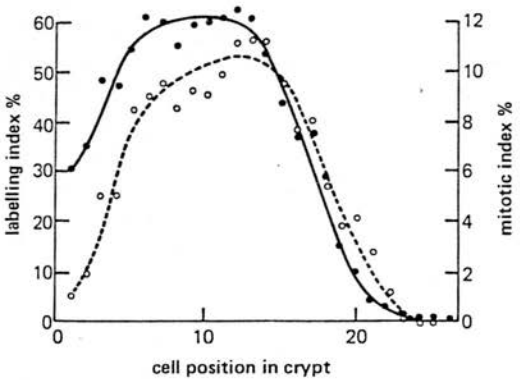

Fig. 3. The labelling and mitotic indices at each cell position in the jejunal crypts of male Balb/c mice. (Al-Dewachi et al., 1975). The labelling index is represented by the *solid* line and circles

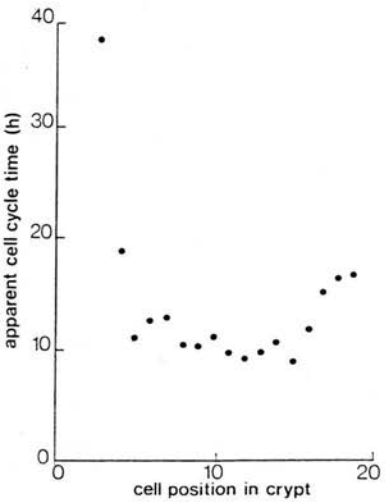

Fig. 4. The apparent cell cycle time at each cell position in the jeunual crypts of male Balb/c mice. measured using the stathmokinetic technique after injection of vincristine (adapted from Al-Dewachi et al., 1975)

such cells are present what is measured will be longer than the cell cycle time derived from the proliferative cells alone by the FLM technique. The calculation of $T_{c(a)}$ is also much more dependent on the assumption of the correct age distribution of cells than is the direct calculation of T_c; Fig. 4 shows the $T_{c(a)}$ values for each cell position on the assumption of an exponential age distribution. No results are given for cell positions 1 and 2 because mitoses failed to accumulate, but $T_{c(a)}$ is clearly prolonged in basal cell positions; one reason for the longer values is the considerable number of Paneth cells seen in the base of the crypt (Al-Dewachi et al., 1975). The apparent cell cycle time is also long at the top of the crypt due to cells decycling as they begin to differentiate, but in the proliferative compartment $T_{c(a)}$ is fairly constant, lying between 10 and 12 h. Since these values are close to the estimates of T_c for the same cell positions the growth fraction in the proliferative compartment must be lose to unity. This is borne out by using the phase times of Table 1 to calculate theoretical labelling indices and comparing with these the observed labelling indices of Fig. 3.

The fit of the computed FLM curves to the data calls for some comment. The curves generally fit the first peaks well, but in some instances they do not fit the second peaks adequately. One reason for this is that the Gilbert program gives more weight to the experimental points on the first peak. However, the second peak for cell positions 15 and 16 and 17+ are examples of what Steel (1977) calls 'fade'. Since there was no difficulty in recognising labelled mitoses, label dilution being negligible, this phenomenon could be due to a loss, maybe through migration, of labelled cells. Inspection of Figure 3 shows that cells begin to decycle above cell position 14 and cell position groups 15 and 16 and 17+ will contain some non-proliferative cells. More difficult to account for is the curve shown for cell positions 7 and 8 where the second peak shows 'enhancement', with most experimental points lying above the fitted curve. There is a problem in the interpretation of FLM curves when cells are migrating. While this factor will not affect results in lower cell positions where migration rates are very low, in higher positions a cell appearing first as a labelled mitosis in position 9, say, may be a labelled mitosis at cell position 16 or even higher by the time of the second peak. Consequently cells which are in quite different parts of the crypt at the time of ^{3}HTdR administration end up providing data for the same FLM curve, and indeed it is not clear what we actually mean by 'the cell cycle time at position k'. However, this consideration should not affect the flavour of our main conclusions: that basally situated crypt cells have extended cell cycle times compared with cells in the proliferative compartment.

The implications of this study and the questions it cannot answer are several: a direct measurement of the cell cycle time in basal crypt cells shows that they are on average cycling more slowly then cells in the proliferative compartment. Cells in the bottom two positions are now regarded as 'functional' stem cells (Cheng and Leblond, 1974; Wright, 1978), indicating that they supply cells to the proliferative compartment in normal circumstances, and we have shown that they resemble the bone marrow stem cells in having long cell cycle times. However, many cells in positions 3–8 also have relatively long cycles compared to the crypt as a whole. Potten and Hendry (1975) have suggested, on the basis of radiobiological studies, that the mouse crypt contains about 80 cells which are capable of crypt repopulation; this is just less than half of the proliferative population of the crypt, and it is possible that these are the cells with long cell cycle times, but it is necessary to be careful in making this link, for 'short' and 'long' are merely convenient relative terms and have no intrinsic meaning.

If our supposition were true then the longer cycling 'potential' stem cells mainly at the base of the crypt would be expected to survive phase specific cytotoxic damage preferentially and be available to repopulate the crypt in the manner shown by Al-Dewachi et al. (1977) in the rat crypt after hydroxyurea. However, experiments in the mouse with cytosine arabinoside, an S-phase-specific agent, show that repopulation occurs from the proliferative compartment as well as the basal crypt cells (Al-Dewachi et al., 1979). It is possible that repopulation mechanisms vary with the number of cells killed, and more work is required before the question of which cells repopulate the crypt under which circumstances can be resolved.

Acknowledgements. This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss P. Barratt who typed the manuscript and Mrs. E. Wallace and Mrs. M. Hughes who provided technical assistance.

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Received November 20, 1978

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Virchows Arch. B Cell Path. 18, 225—242 (1975)
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Cell Population Kinetics in the Mouse Jejunal Crypt

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Received March 17, 1975

Summary. The time parameters of the cell cycle were determined in the jejunal crypts of male Balb/c mice by an FLM experiment. The cell cycle time was 12.42 ± 0.11 h, and the duration of DNA synthesis was 7.61 ± 0.09 (mean values $\pm$ S.E.). A stathmokinetic technique using vincristine gave a value of T_c of 11.8 h.

The growth fraction (I_p) calculated from cycle parameters and the labelling index was 0.61, while a value of I_p estimated from a labelling index distribution curve was 0.65.

For the whole crypt a value of 0.86 h was obtained for the duration of mitosis, longer than that in the rat. The mitotic duration was found to vary with cell position, but values of between 0.8 and 1.2 h prevailed throughout the proliferative compartment; values in the basal cell positions appeared shorter. Apparent cell cycle times were longest in the basal cell positions.

A value for crypt migration rate calculated from a cumulative birth rate curve was 1.48 cell positions per h, compared with a value of 1.8 ± 0.27 cell positions per h as measured from movement of the 50% peak value on the labelling index distribution curve with time after tritiated thymidine.

The crypt cell production rate was calculated from microdissected and squashed crypts to be about 14 cells per crypt per h. There was a total crypt population of 282 ± 65 (S.E.) cells, of which 172 were proliferative.

Key words: Intestine — Mitosis — Cell Kinetics — Thymidine.

Introduction

Because it is small and convenient in use, the mouse has been extensively employed as an experimental model for studying cell kinetics in the small bowel mucosa, particularly in the comparison of different kinetic situation (for example, Lesher, 1967). Other workers have been concerned with the intrinsic significance of kinetic events in the mouse. The pioneering studies of Quastler and Sherman (1959) rationalised cell population kinetics in general, and these workers attempted a kinetic analysis of the renewal system in terms of size, flux and time. Thrasher and Greulich (1965a, b; 1966) carried out a spatial analysis of the mouse crypt which to some extent confirmed the conclusions of Cairnie, Lamerton and Steel (1965a) in the rat. Recently a new approach to small bowel kinetics in the mouse has been evolved. This entails crypt microdissection and scintillation counting with tritiated thymidine (^{3}HTdR) (Hagemann, Sigdestad and Lesher, 1970; Kovacs and Potten, 1973).

Since a combined study using ^{3}HTdR labelling techniques in association with stathmokinetic studies with vincristine proved applicable in the rat smalo bowel (Wright, Morley and Appleton, 1972a; Al-Dewachi, Wright, Appleton anld Watson, 1974; Wright, Al-Dewachi, Appleton and Watson, 1974) a similar study was undertaken in male Balb/*c* mice. It is important to compare the results with

previously published values for the mouse, and comparison of kinetic parameters as between the mouse and the rat shows some interesting differences.

Materials and Methods

Male Balb/c mice were used throughout. The animals were aged 3 months and weighed 20–25 g. All injections were carried out at 0900 h in an attempt to minimise the effects of diurnal variation of proliferative indices (Sigdestad, Bauman and Lesher, 1969).

A. ^{3}HTdR Studies

(i) FLM Experiment. Forty nine animals were injected with ^{3}HTdR (Radiochemical Centre, Amersham, England) in a dosage of 1 μCi per g body weight, given by the intraperitoneal route. The concentration of ^{3}HTdR used was 100 μCi per ml. Animals were killed serially thereafter by cervical dislocation, and samples of upper jejunum were removed immediately distal to the ligament of Treitz. The tissue was fixed in Carnoy's fluid for 6 h, embedded in paraffin and 3 μm sections were processed for autoradiography as previously described, the exposure period being 14 days (Al-Dewachi *et al.*, 1974).

In each animal the percentage of labelled mitoses was determined by counting at least 100 metaphases and anaphases; this restriction was imposed to avoid the difficulties experienced in the identification of labelled prophases and telophases cut in section. The incidence of labelled mitoses was plotted against time, and the mean durations of cycle parameters were measured by analysing the data by the method of Gilbert (1972).

(ii) Proliferative Index Distribution Curves. Five animals were killed 1 h after the injection of ^{3}HTdR, and autoradiographs prepared from the upper jejunum. In each animal 30 axially sectioned crypts were analysed, and the cell positions in the left hand column, of labelled and mitotic nuclei were recorded, using the counting methods of Cairnie *et al.* (1965a). The data from these five animals were combined, and labelling and mitotic index distribution curves were constructed. The variation in the distribution of labelled and mitotic cells as between long and short crypts was compensated for by analysing the data by the computer program of Cairnie and Bentley (1967) as modified by Wright *et al.* (1972a).

In the animals killed up to 4 h after ^{3}HTdR in the FLM experiment, 30 axially sectioned crypts were selected and the cell positions of labelled nuclei were recorded. Labelling index distribution curves were drawn for each animal, and the cell positions of the 50% peak value determined.

The criterion for the identification of labelled cells was 5 or more grains localised over the nucleus (Wright, 1971).

B. Vincristine Studies

Fifteen animals were used in this experiment. Three uninjected control mice were killed at 0900 h. The remaining animals were injected with 1 mg of vincristine sulphate (Oncovin, Lilly) per kg body weight, given by intraperitoneal injection. One animal was killed every 15 min for 2.5 h. At 2.5 h a further two animals were killed. Samples of upper jejunum were obtained from the same site, fixed in Carnoy's fluid for 6 h and embedded in paraffin. Sections were prepared at a thickness of 3 μm and stained with Harris's haematoxylin.

One hundred axially sectioned crypts were selected in each animal, and the cell positions of mitotic nuclei in the left hand crypt column recorded. In the untreated control animals all stages of mitosis were counted; in the animal killed at 15 min after vincristine injection some telophases were apparent, but at 30 min and thereafter only prophases and metaphases were seen. The criteria for the recognition of mitotic stages were those of Clarke (1970). The data were analysed by the computer program, and mitotic index distribution curves constructed for each animal.

Tannock's constant (Tannock, 1967) was estimated from analysis of 200 approximately circular crypt sections containing metaphases. These sections were prepared by cutting tangentially to the bowel lumen and the radial distances from the crypt axis of metaphase and interphase nuclei were measured using a calibrated eye piece graticule.

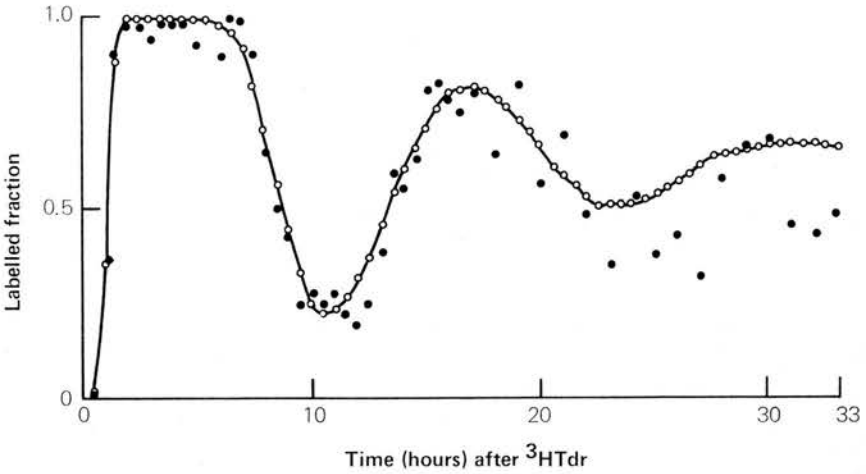

Fig. 1. FLM curve for the jejunal crypt of male Balb/c mice. The closed circles are the experimental points, while the open circles are the points and the line fitted by the Gilbert program

In the three uninjected control animals tangential sections were also prepared, and the number of cells in crypt transverse sections, *the column count*, was measured in 100 approximately circular cross-sections in each animal.

To exclude non-proliferative Paneth cells from calculations in basal cell positions, sections stained with haematoxylin and eosin were prepared from each of the three control animals. In each animal 100 axially sectioned crypts were analysed for Paneth cells. The crypts were standardised and the percentages of Paneth cells were calculated for each cell position.

(iii) Crypt Microdissection. Three animals were killed at 0900 h and samples of upper jejunum were prepared for crypt microdissection following the technique of Clarke (1971). The hydrolysis was carried out at 60° in N/1 HCl for 6 min before bulk staining with Schiff's reagent. In each animal 10 crypts were dissected, squashed under a coverslip and the number of cells per crypt and the number of mitoses per crypt were counted.

Results and Interpretation

FLM Data

Fig. 1 shows the FLM curve obtained for the mouse jejunum. Definitely two peaks and possibly the beginning of a third peak may be seen. The closed circles are the observational points and the open circles and the line are the computed points and line respectively. Over the first two peaks the computed line describes the data well, but in the second trough the computed curve flattens before the experimental data points.

Table 1 gives the computed mean values for the duration of cycle parameters with standard errors, and coefficients of variation. The coefficient of variation for the cell cycle time (T_c) is quite high, indicating a considerable spread in the distribution of individual cell cycle times, but the coefficient of variation of t_s+tg_2 is comparatively small, and hence smaller variations would be expected between individual values of the duration of DNA synthesis, t_s. The mean value of $T_c(\pm$S.E.) is 12.42 ± 0.11, and the mean t_s is 7.61 ± 0.09.

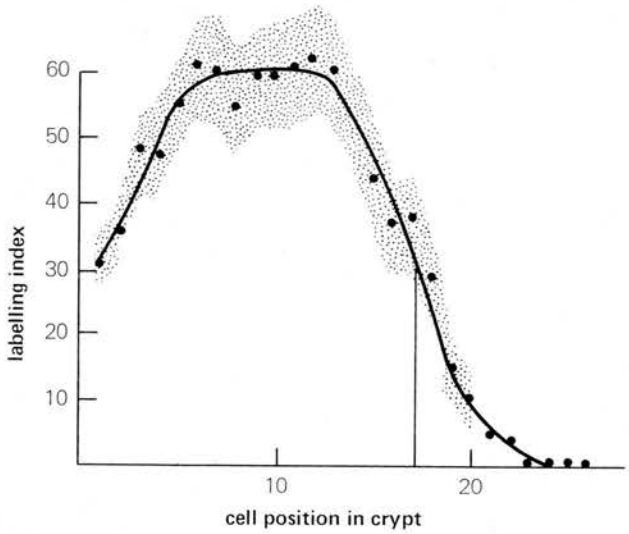

Fig. 2. The labelling index distribution curve for the mouse jejunal crypt. The shaded area indicates 95% confidence limits for the points

Table 1. Phase durations for the jejunal crypts of male Balb/c mice (h)

	T_c	t_s	t_{g2}	t_{g1}	t_m	CV_c (%)	CV_{g2} (%)	CV_{g2+s} (%)
Mean ± S.E. (Gilbert program)	12.42 ±0.11	7.61 ±0.09	1.15 ±0.03	3.66 ±0.13	—	21.23	28.16	14.90
Stathmokinetic (vincristine)	11.8	—	—	—	0.86			

The Gilbert program regards mitosis as an instantaneous event, and thus tg_1 and tg_2 will be overestimated. A value of t_m could be given by the FLM curve, since the time from injection of ^{3}HTdR to the 50% peak value on the upswing of the first peak is equal to the median $t_{g^2}+\frac{1}{2}t_m$, i.e. t_2. Even though readings were made here at 30 min intervals, because of the steep inclination there are few experimental points on this portion of the curve, and an accurate measurement of t_2 would need many more readings; these animals are better employed in a stathmokinetic experiment to measure t_m.

The Proliferative Index Distribution Curves

Fig. 2 shows the labelling index distribution curve drawn from the data provided by analysis of the five pulse-labelled animals killed after 1 h exposure to ^{3}HTdR. Comparatively low I_L values are evident in the first two cell positions, but the labelling index rapidly increases to reach a plateau value of over 60%, beginning at cell position 6 and ending at position 13. A steady fall then occurs over the next seven cell positions to reach low levels of around 5% at cell position 21, and thence to zero in the last few cell positions. The mean number of cells in the standardised crypt column was 26.

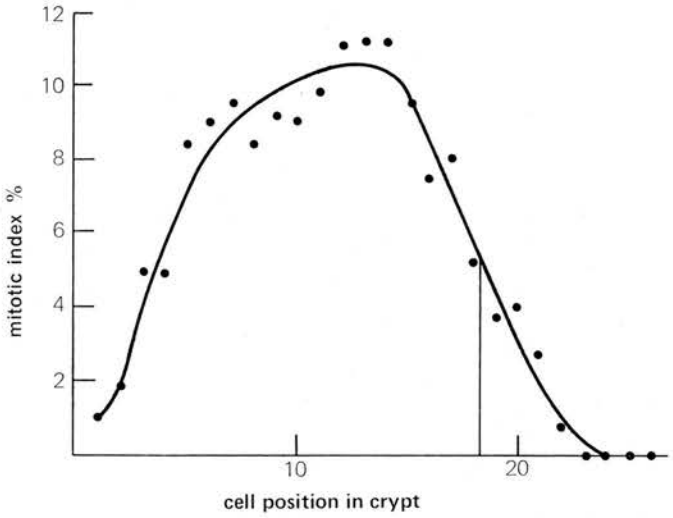

Fig. 3. The mitotic index distribution curve for the mouse jejunal crypt

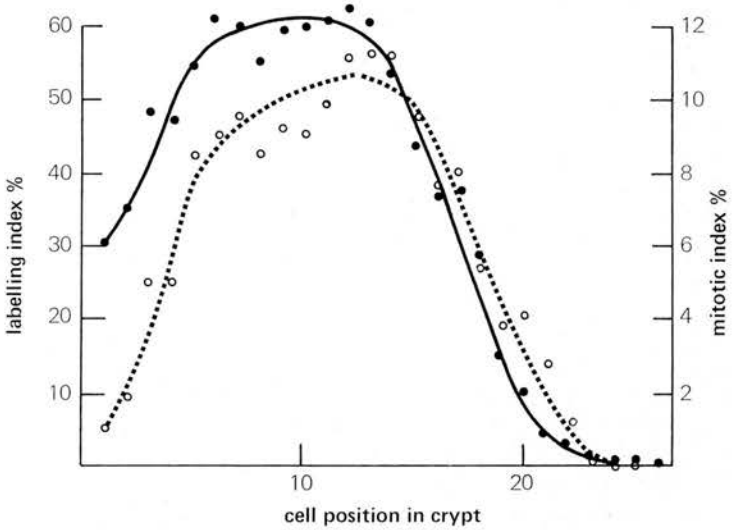

Fig. 4. The labelling index distribution curve (●) compared with the mitotic index distribution curve (○) for the mouse jejunal crypt

The theoretical labelling index, calculated assuming steady state conditions from the ratio of t_s to T_c is realised at the plateau value within the proliferative compartment (Cleaver, 1967); we conclude that few non-proliferative cells exist within this portion of the crypt, assuming that the cycle parameters as measured for the whole crypt apply equally to this portion of the crypt.

The mitotic index distribution curve is shown in Fig. 3. Here very low values for I_m are apparent in the basal positions, followed by a slow rise to peak values of about 11% in cell positions 12–14. There follows a less precipitous fall than was

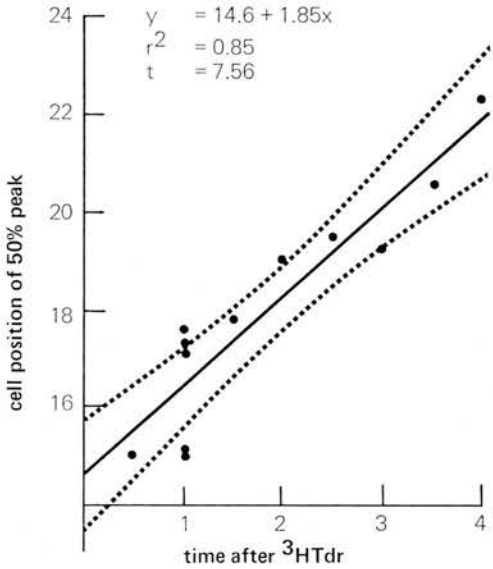

Fig. 5. Movement of the 50% peak value on the labelling index distribution curve with time after ^{3}HTdR. The broken lines indicate 95% confidence limits for the line

evident in the labelling index distribution curve; in the apical cell positions the mitotic index curve falls slightly to the right of (i.e. above) the labelling index curve (Fig. 4).

Proliferating Populations

A growth fraction (I_p) for the whole crypt may be calculated from the expression

$$I_p = \frac{I_{L\,\text{expt.}}}{I_{L\,\text{theor.}}} \quad (1)$$

where $I_{L\,\text{expt}}$ is observed labelling index and $I_{L\text{theor.}}$ is the theoretical labelling index calculated, assuming steady state conditions, from the ratio of t_s to T_c. Schultze, Haack, Schmeer and Maurer (1972) have shown that steady state conditions do apply to the mouse crypt considered as a whole. The experimental index is 37% and $I_{L\,\text{theor.}}$ was calculated above as 61%. I_p is 0.37/0.61 or 0.61.

Cleaver (1967) proposed that, in the terms of the crypt column, the proliferative compartment could be approximately defined by noting the cell position of the 50% peak on the labelling index distribution curve; the ratio of the number of cell positions in this "proliferative compartment" to the number of cells in the crypt column gave values for I_p which compared well with estimates from cycle parameters (Cleaver, 1967; Wright, Morley and Appleton, 1972b; Wright *et al.*, 1974). Here the 50% peak is at cell position 17; therefore I_p for the whole crypt is $^{17}/_{26}$ or 0.65, agreeing well with the previous value of 0.61.

Movement of the 50% Peak Value with Time

The movement of the 50% peak value with time is shown in Fig. 5, and a good degree of linearity is achieved over the experimental period with a r^2 value of

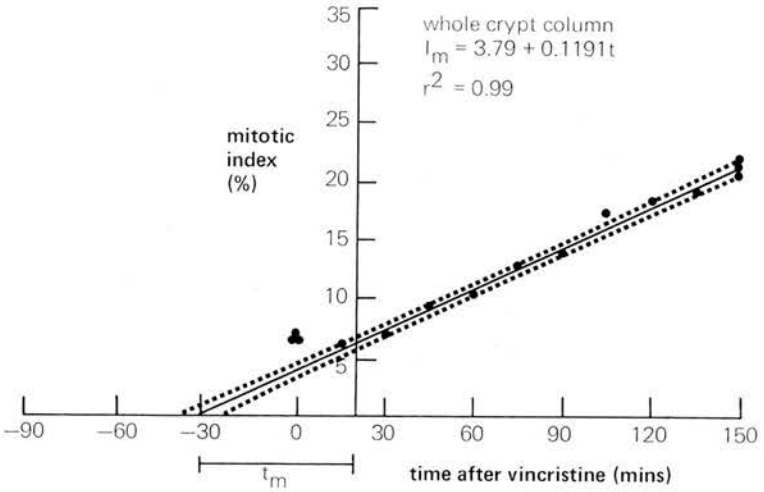

Fig. 6. Mitotic index accumulation line for the whole crypt column with time after vincristine. The broken lines indicate 95% confidence limits for the line

0.85. After 4 h labelled cells began to appear on the villi. The slope of the line is a measure of the migration rate towards the top of the crypt, and is 1.85 ± 0.27 cell positions per h.

Stathmokinetic Results

Fig. 6 shows the mitotic accumulation with time after vincristine for the whole crypt. The graph shows a very good degree of linearity over the experimental period with an r^2 value of 0.99. However, there is an important and interesting difference between this present line and the vincristine accumulation line obtained for the rat (Wright *et al.*, 1972a). There is a delay of some 20 min before metaphases begin to accumulate. The presence of such a delay period may be related to the concentration of the stathmokinetic agent (Taylor, 1965, and see discussion). Calculation of the slope from all readings, including those at zero time would give undue emphasis to these early readings. For this reason it has been assumed that vincristine shows a delay period of 20 min; the readings at zero time have also been disregarded in the regression analysis for calculating the line of best fit (see discussion).

The Cell Cycle Time

The slope of the line is a function R of the rate of entry into mitosis and is 7.15% cells/h; Tannock's constant is 0.73 which is larger than the value obtained for the rat (0.62). The growth fraction is 0.61. Consequently r, the rate of entry of cells into mitosis per proliferating cell of the population is

$$r = \frac{7.15}{0.61 \times 100} \times 0.73 = 0.085.$$

Again assuming steady state conditions the mean T_c is

$$T_c = \frac{1}{r} = \frac{1}{0.085} = 11.8 \text{ h}$$

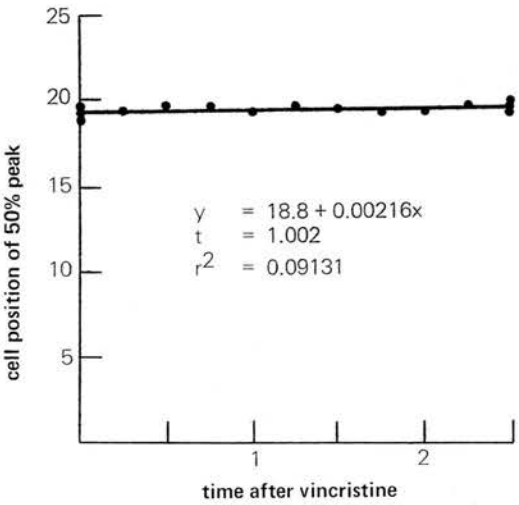

Fig. 7. Movement of the 50% peak value on the mitotic index distribution curve with time after vincristine

which is in reasonable agreement with the mean T_c of 12.42 h measured from the FLM curve.

Mitotic Duration

As there is evidently a delay in the onset of metaphase arrest, calculating t_m from the equation

$$t_m = \frac{I_{m(o)}}{R} \tag{2}$$

(Wright *et al.*, 1972a), where $I_{m(o)}$ is the mitotic index at zero time, would again give undue emphasis to these early readings; consequently we have used the method in which the regression line is extrapolated backwards to cut the x-axis. The negative intercept on the x-axis is then t_m. This method is particularly applicable when a delay in metaphase arrest is evident; in this case, we have to add the delay period to the negative intercept to find t_m (Brown and Berry, 1968). The mitotic duration for the whole crypt column is 0.86 h (95% confidence limits 0.75 h and 0.99 h).

Variation in t_m with Cell Position

If no movement of the arrested metaphases should occur with respect to the interphases we may measure t_m at each cell position in the crypt column. This point was checked by plotting the cell position of the 50% peak value against time after vincristine. The movement of the 50% peak with time is shown in Fig. 7; the line is not significantly removed from the horizontal, and we may conclude that no effective movement of arrested metaphases occureed during the experimental period.

Mitotic accumulation lines were drawn for each cell position and a selection of regression lines from various positions is shown in Fig. 8. The r^2 values for each

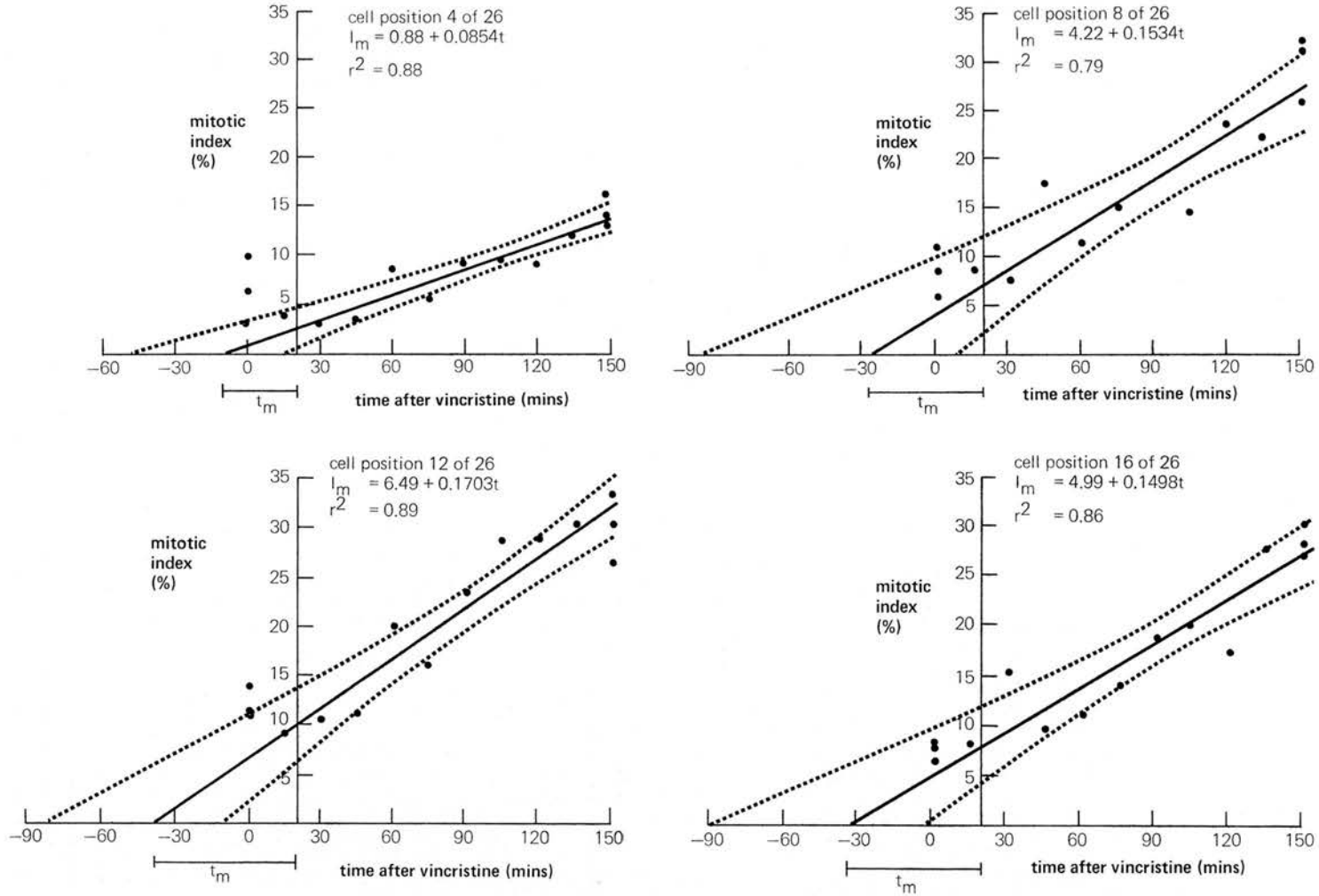

Fig. 8. Mitotic index accumulation lines for some cell positions in the crypt column with time after vincristine. The broken lines indicate 95% confidence limits for the lines

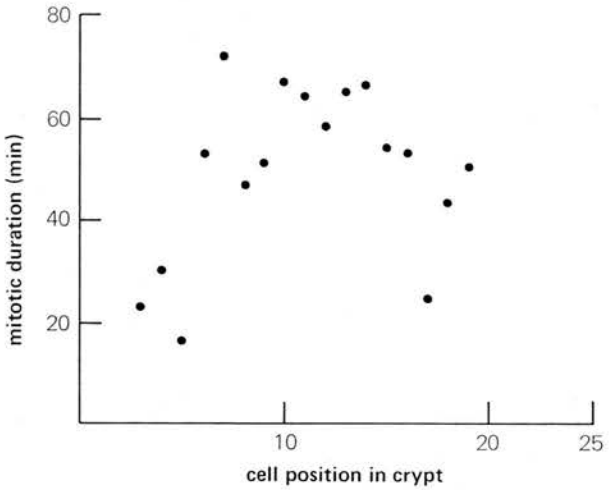

Fig. 9. Variation in the duration of mitosis with cell position in the mouse jejunal crypt

cell position are shown in Table 2. The data begins at cell position 3; the lines for positions 1 and 2 were not significantly different from the horizontal. The reasons for this are several. The percentage of Paneth cells is high in these cell positions—the number of intercalated cells is therefore very few in relation to the relatively large numbers of non-proliferative Paneth cells. This would not matter if these intercalated cells proliferated rapidly. However, apparent cell cycle times ($T_{c(a)}$) for cell positions 3 and 4 are long compared with the proliferative compartment. A combination of low proliferative rates and a low growth fraction is no doubt responsible for the absence of a rise in metaphase index; extension of the duration of the stathmokinetic period would invite large errors due to metaphase degeneration (Aherne and Camplejohn, 1972).

Although a low r^2 value is obtained at position 3, values of above 0.7 and in most cell positions above 0.8 are evident among the remaining cell positions. Above cell position 20 the accumulation was most irregular, with no distinct linear trend. This is probably due, not to any decrease in proliferative rates, but to a fall in growth fraction as cells decycle. The variability in crypt length may be a further contributory factor, notwithstanding the crypt standardisation procedure.

A value for t_m was calculated for each cell position and the data is presented in Fig. 9; the individual values with 95% confidence limits are given in Table 2. The duration of mitosis is apparently shorter in lower cell positions compared with the remaining proliferative cells; however, the confidence limits are large, particularly in cell position 3. In the remaining cell positions the values are between 0.8 and 1.2 h, except in cell position 17, where the value is 0.4 h. A mean value calculated for positions 4–18 is 0.86 h (confidence limits 0.73 h and 1.00 h).

Table 2. A summary of the stathmokinetic data for each cell position (r^2 values refer to the regression line obtained with vincristine)

Cell position	3	4	5	6	7	8
r^2	0.58	0.88	0.94	0.88	0.72	0.79
t_m (h)	0.39	0.50	0.27	0.89	1.20	0.79
low C.L. t_m	—	0.1	—	0.4	0.4	0.2
high C.L. t_m	3.7	1.2	0.6	1.7	3.2	2.0
$T_{c(a)}$ (h)	55	27	15.9	17.8	18.3	14.9
Cell position	9	10	11	12	13	14
r^2	0.77	0.92	0.91	0.89	0.87	0.84
t_m (h)	0.85	1.11	1.66	0.97	1.08	1.10
low C.L. t_m	0.2	0.7	0.6	0.5	0.5	0.5
high C.L. t_m	2.2	1.7	1.7	1.7	2.0	2.1
$T_{c(a)}$ (h)	14.9	15.8	13.4	13.1	13.9	15.0
Cell position	15	16	17	18	19	
r^2	0.93	0.86	0.90	0.90	0.73	
t_m (h)	0.90	0.89	0.40	0.7	0.83	
low C.L. t_m	0.5	0.6	—	0.3	0.2	
high C.L. t_m	1.4	1.7	0.9	1.4	2.4	
$T_{c(a)}$ (h)	12.7	16.7	21.4	23.1	23.4	

Variation in Apparent Cell Cycle Time $T_{c(a)}$ with Cell Position

The rate of entry into mitosis R plotted as a function of cell position in the crypt is shown in Fig. 10; the shaded area indicates 95% confidence limits for the points. Lowest values are seen in positions 3 and 4, followed by a rise to peak rates in cell positions 11–15; the rate of entry into mitosis then falls in the remaining cell positions studied. $T_{c(a)}$ values, calculated from the reciprocal of the rate of entry into mitosis, and assuming that the value obtained for Tannock's constant is applicable at all cell positions, are given in Table 2, and plotted as a function of cell position in Fig. 11. In positions 3 and 4 the values of $T_{c(a)}$ are longer, falling to between 12 and 18 h in the higher cell positions. There may be further lengthening of $T_{c(a)}$ towards the top of the crypt.

Paneth Cell Distribution

Percentages of Paneth cells at each cell position are shown in Fig. 12. In position 1 of the standardised crypt nearly 80% of cells are Paneth cells, reducing to 60% at cell position 2. Metaphase accumulation data was not obtainable for cell positions 1 and 2, and $T_{c(a)}$ values for positions 3 and 4 will obviously be affected by the still considerable number of Paneth cells present; $T_{c(a)}$ at cell position 3 drops from 55 h to 31 h when Paneth cells are excluded from the population, and at position 4 from 27 to 21 h. Even so, $T_{c(a)}$ at position 3 remains longer than in the remaining cell positions. It should be remembered that Tannock's constant is assumed to be the same at all cell positions, but this may not be justified in the lower positions.

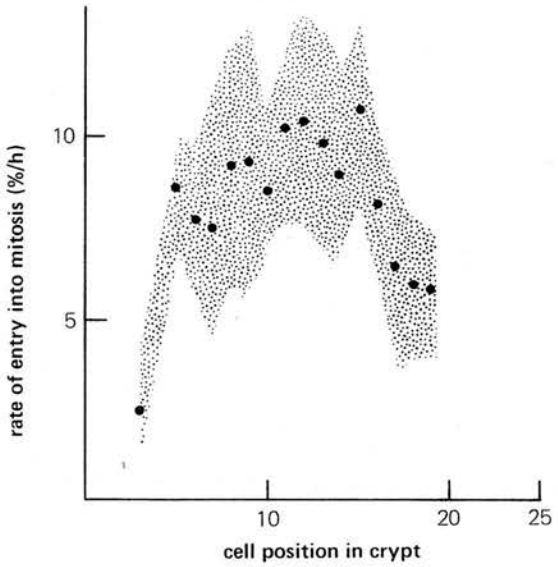

Fig. 10. The rate of entry into mitosis (% cells/h) as a function of cell position in the crypt. The shaded area indicates 95% confidence limits for the points

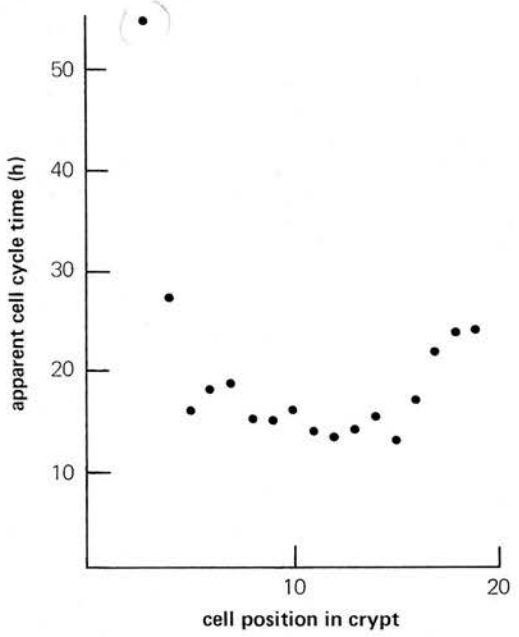

Fig. 11. Variation in apparent cell cycle time ($T_{c(a)}$) with cell position in the mouse jejunal crypt

As in the rat, $T_{c(a)}$ in lower cell positions is prolonged (Wright *et al.*, 1972a), while in the mouse only position 3 shows a prolonged $T_{c(a)}$ when Paneth cells are excluded. No kinetic data is available for positions 1 and 2 for reasons discussed

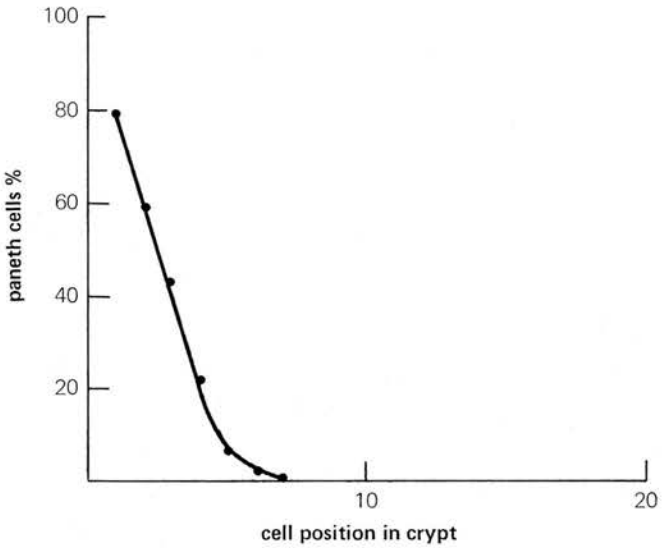

Fig. 12. Population percentages of Paneth cells at each position in the mouse jejunal crypt

above, and it may be that these cells share the extended $T_{c(a)}$ values recorded for position 3. If I_p here is unity, cell cycle times are prolonged and the positions may be "stem cells" for the above proliferative compartment.

The Cumulative Birth Rate

The birth rate k_b was calculated for each cell position from the equation

$$k_b = \frac{I_m}{t_m} \tag{2}$$

The values were added and the cumulative birth rate curve is shown in Fig. 13. The choice of t_m is important in the calculation of such curves (Wright *et al.*, 1972a) and t_m values for each cell position measured from the mitotic accumulation curves have been used. The maximum value attained is 1.48; if cell movement within the crypt is a function of proliferative activity, then this is equal to a migration rate of 1.5 cell positions per h. In terms of the crypt column, it is also equal to the efflux onto the villus.

Crypt Population

The crypt column length was 25.1 ± 0.2 (S.E.) cells, while the column count was 14.5 ± 0.2 (S.E.) cells. The estimate of the total crypt population obtained from the product of these values (Wimber and Lamerton, 1963) is 366 cells. Direct counting of total cells in the squashed microdissected crypts gave a value of 282 ± 65 cells per crypt, while the mitotic index was 7.26 ± 0.63 % (see discussion).

An estimate of total proliferating population is available from the product of the crypt population and the growth fraction, and is 172 cells per crypt from the crypt squash data, and 223 cells as calculated from morphometric parameters. The two methods give 100 and 143 non-proliferative cells per crypt respectively.

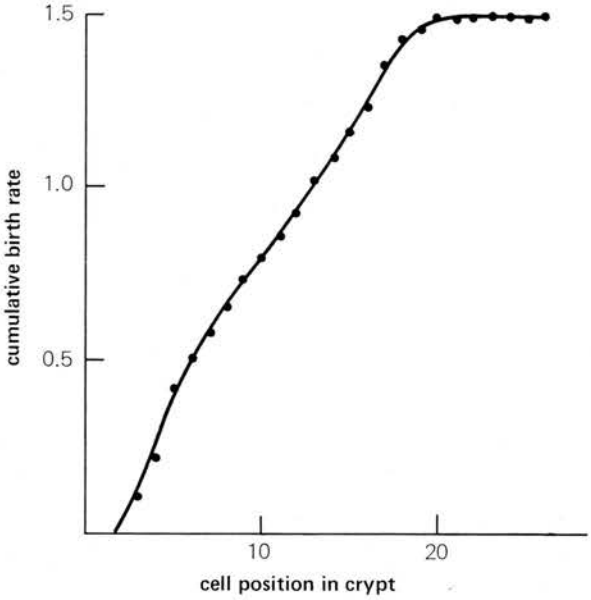

Fig. 13. Cumulative birth rate curve for the mouse jejunal crypt

Flux Parameters

The crypt cell production rate can be calculated from the present data. In terms of the whole crypt, the birth rate k_b is the efflux from the proliferative compartment, or effectively the efflux from the crypt, and

$$k_b = \frac{I_m}{t_m} \times N_p \tag{3}$$

where N_p is the number of proliferative cells per crypt. This gives a value of 14.5 cells per crypt per h, using the crypt squash data. We may also calculate k_b from

$$k_b = \frac{I_p}{T_c} \times N_c \tag{4}$$

where N_c is the number of crypt cells, and again assuming steady state conditions. This gives 13.8 cells per crypt per h, in good agreement with the above value.

If the peak value achieved in the cumulative birth rate curve is the efflux onto the villus per crypt column, then the product of this and the number of crypt columns will give a value for the total efflux, assuming a cylindrical crypt. This is 14.5×1.48, or 21.4 cells per crypt per h, which does not compare so well with the previous values.

The total crypt transit time calculated by summation of the reciprocals of the individual birth rates at each cell position (Cairnie *et.al.*, 1965a; Wright *et al.*, 1974) is 24.8 h, if the prolonged values for positions 3 and 4 are excluded. The minimum time taken for villi to become labelled after injection of ^{3}HTdR was

about 4 h, which agrees with the t_{g^1}' of Quastler and Sherman (1959), who probtook as their criterion the first labelled cells to reach the villus (Cairnie *et al.*, 1965a). This value represents a minimum time for the transit of a labelled cell through the maturation sequence. Extrapolation of the graph describing the movement of 50% peaks (Fig. 5) to the standardised crypt length will give the time taken for the 50% peak to reach the foot of the villus, or the median transit time through the maturation sequence (Cleaver, 1967), which is 6.2 h. The minimum transit time from the final S in the proliferative compartment is thus 6.2 $(t_m \mid t_{g^2})$ or 4.9 h. The median time taken for a completely non proliferative cell to traverse the maturation sequence is $6.2-(0.5t_s+t_{g^2}+t_m)$ or about 1.1 h (Cairnie *et al.*, 1965a). Here median values have been used for the duration of cycle parameters, since the mean values given by the Gilbert program do not include t_m, and thus exaggerate mean t_{g^2} values.

It is not possible to estimate stem cell efflux using the hypothesis that basal cell positions with longer $T_{c(a)}$ values are stem cells, since data for positions 1 and 2 are not available. However, the transit time from cell positions 5–22 (here excluding possible stem cells and definitely non proliferative cells at the top of the crypt) is 22 h, and with T_c at about 12 h the number of transit divisions in the proliferative compartment will be about 2.

The kinetic parameters are summarised in Table 3.

Table 3. A summary of kinetic parameters

Crypt column length (cells ± S.E.)	Column count (cells ± S.E.)	Crypt population (cells)	Number of proliferative cells/crypt	Number of non-proliferative cells/crypt	Growth fraction
25.13 ± 0.24	14.55 ± 0.20	282 ± 65[a] 366[b]	172[a] 223[b]	100[a] 143[b]	0.61[c] 0.65[d]

Migration rate (cell positions/h)	Crypt cell production rate (cells/crypt/h)	Crypt transit time (h)	Maturation compartment transit time (h)
1.48[e] 1.85 ± 0.27[f]	14.5[g] 13.8[h] 21.5[i]	24.8[j]	4.9[k] 1.1[l]

[a] From microdissected squashed crypts.
[b] From product of crypt length and column count.
[c] Calculated from phase durations and I_L.
[d] Measured from the labelling index distribution curve.
[e] Read from the cumulative birth rate curve.
[f] From movement of 50% peak values on the labelling index distribution curve with time.
[g] Microdissected crypt population and $k_b = I_m/t_m$.
[h] Microdissected crypt population and $k_b = I_p/T_c$.
[i] Product of migration rate (e) and column count.
[j] Summation of reciprocals of birth rates at each cell position.
[k] Minimum transit from final S in proliferative compartment.
[l] Median time for non-proliferative cell.

Discussion

The values obtained for T_c from the FLM and vincristine experiments are similar, and suggest that the same parameter is being measured in each case. The value obtained is however shorter than that derived by Kovacs and Potten (1973), although our t_s is very close to the mean t_s computed from published values by the same authors. The t_m of 0.86 h derived for the whole crypt is most interesting. Wright *et al.* (1972a) found a value of 0.43 h in the rat, and speculated about possible species differences; speculations now apparently justified. The present t_m value of 0.86 h does not support the 0.4 h found by Schultze *et al.* (1972) by calculation from ^{3}HTdR studies, but agrees more with the 1.05 h reported by Pelc (1971) using colcemid.

The present estimates of total crypt population obtained from squashed microdissected crypts of 282 cells compare well with previously reported values for the mouse. Kovacs and Potten (1973) obtained a value of 250 cells per crypt while Hagemann *et al.* (1970) reported a value of 290 cells per crypt. These estimates are higher than that reported by Schultze *et al.* (1972) who counted 174 cells per crypt; all these authors used crypt microdissection. The value derived from morphometric parameters measured in sections is comparatively high at 366 cells — another estimate also carried out on sectioned material (Devik, 1971) led to a high value of 671 cells per crypt for WLO mice. As in the rat, measurements from sections are higher than in squashed microdissected crypts (Wright *et al.*, 1974).

The I_p as calculated from cycle parameters and labelling index was 0.61 and, as in the rat, agreed well with an estimate (0.65) derived from the labelling index distribution curve (Wright *et al.*, 1974). These values are close to that of Hagemann *et al.* (1970a) who found I_p to be 0.59, but somewhat higher than that of Kovacs and Potten (1973) who reported a value of 0.54, and of Fry, Lesher, Kisieleski and Sacher (1963) and Lesher (1967) whose data have been analysed by Kovacs and Potten (1973) to give a value of 0.55. Fry, Lesher and Kohn (1961) and Schultze *et al.* (1972) reported higher values of 0.70 and 0.75 respectively. The similarity between the present estimate for the mouse jejunal crypt and that derived for the rat (Wright *et al.*, 1974) is quite striking.

From the crypt population in squashed crypts and the growth fraction, an estimate of 172 proliferating cells per crypt was found, agreeing well with the 171 cells per crypt obtained by Hagemann *et al.* (1970). Kovacs and Potten (1973) recorded 135 proliferative cells per crypt, and calculated a value of 125 cells per crypt from the data of Lesher (1967); this would be expected as crypt population and I_p were lower in these latter studies. A smaller crypt population is also responsible for the lower estimate of Schultze *et al.* (1972) who found only 99 proliferative cells per crypt. Differences in strain and in technique may account for this variation. The number of non-proliferative cells per crypt at 110 is lower than that found by Hagemann *et al.* (1970), 143 cells per crypt.

No differences were apparent in crypt population either between mice or within the sample from each mouse. However, in the case of the mitotic index, a significant difference was found between mice ($F_{2127}=4.410$, $p<0.05$, residual standard deviation 0.27). For simplicity in calculating the crypt cell production rate we have therefore taken the mean value of the mitotic index readings.

The distribution curve for Paneth cells at the base of the crypt shows considerable differences compared with the rat—the Paneth cell compartment is much larger, 8.9% compared with 0.8% in the rat. Lesher (1967) also found about 10% of crypt cells to be Paneth cells in the mouse. Kovacs and Potten (1973), studying incompletely squashed crypts, found that 19% of crypt cells were in the Paneth cell region; this portion of the crypt showed a smaller labelling index when compared with the "central" or proliferative portion of the crypt, but it is evident that proliferative intercalated cells are present. Vincristine data were not available for positions 1 and 2 for reasons given above, and the kinetic organisation at the base of the mouse crypt must show some differences compared with the rat.

It is difficult to explain why t_m values are shortest in basal cell positions where T_c appears longest—in most positions values between 0.8 and 1 h were apparent. In the mouse 1 mg per kgm body weight of vincristine is insufficient to arrest small bowel cells already in metaphase, which may be related to effective concentration (Taylor, 1965). We have therefore assumed a delay period of 20 min.

In the mouse the value for the migration rate of 1.8 cell positions per h given by the movement of 50% peak values compared with the value of 1.48 cell positions per h evident from the cumulative birth rate curve. In the rat the cumulative birth rate gave a higher value than did the movement of 50% peak (Wright *et al.*, 1974).

Calculation of the crypt cell production rate from crypt squash data and stathmokinetic results on the one hand and crypt squash data and FLM results in the other, gives similar values of 13.6 and 13.8 cells per crypt per h respectively. These estimates agree well with that reported by Hagemann *et al.* (1970) of between 13 and 15 cells per crypt per h. Calculation from the migration rate and the column count gives a more disparate value than in the rat (Wright *et al.*, 1974), and the assumptions here involved may not be justified.

The proliferative index distribution curves shown in Fig. 4 is supportive evidence for a kinetic organisation similar to that proposed by Cairnie, Lamerton and Steel (1965b) for the rat; definitive evidence awaits the demonstration of constant T_c and t_s values at the top of the crypt.

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Virchows Arch. B Cell Path. 26, 275–287 (1978)

Virchows Archiv B
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Cell Population Kinetics in the Epithelium of the Colon of the Male Rat

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Summary. The present study was undertaken in order to try to define some of the kinetic parameters in the colonic mucosa of normal Wistar rats.

Preliminary observations showed considerable morphological differences in the mucosa from site to site along the length of the colon. In particular the height of the crypts (measured in cells) was variable. In addition labelling index studies demonstrated dramatic variations in the distribution of labelling along the length of the crypts from site to site in the bowel.

A single site in the descending colon was selected for more detailed study using a stathmokinetic agent, vincristine, and the continous labelling technique with tritiated thymidine. The results of these investigations suggest that there exists at the base of the crypt a subpopulation of cells cycling more slowly than the cells in the rest of the proliferative compartment. Growth fraction appears to fall with rising cell positions within the crypt.

Key words: Colon – Kinetics – Thymidine – Vincristine.

Introduction

Cell population kinetics of the colonic epithelium has been less extensively studied than that of the small bowel; however Messier and Leblond (1960) using a radioactive thymidine label and microautoradiographic techniques found in rats and mice "numerous reactions just below the middle of the crypts of the ascending colon in the six hours following injection, with only rare reactions at the bottom". They classified the colonic epithelium as a "renewing population". Lipkin and Quastler (1962) working with mice, demonstrated a similar distribution of label through the crypt. They constructed a fraction of labelled mitoses (FLM) curve based on whole crypt data and from this calculated a mean "generation time" for crypt epithelium of the order of 16 h. They further postulated a subpopulation of slowly cycling basally situated cells.

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0340-6075/78/0026/0275/$02.60

Sawicki and Rowinski (1970) derived a cell cycle time of approximately 20 h from FLM experiments in guinea pigs. They concluded that the proliferating pool in the upper part of the crypt was smaller than that in the lower part, but that the cell cycle times of the proliferating cells were similar in these two sites. Sawicki, Rowinski and Blaton (1971) described labelling index distribution curves which showed relatively low labelling indices in the basally situated cell positions, with higher values in the rest of the lower half of the crypt, which then fell to zero through the upper half of the crypt.

In mice, Kovacs and Potten (1973) calculated a cell cycle time for colonic crypt epithelium of 23.2 h. This value was derived from flash labelling studies of squashed whole crypts, and values for the duration of DNA synthesis (t_s) derived from the literature. A rectangular age distribution was assumed. Potten, Kovacs and Hamilton, 1974, on the basis of continuous labelling data reported a cell cycle time of 18.2 h for the "proliferative zone" of the mouse colonic crypt.

Tutton and Barkla (1976) used a stathmokinetic technique to calculate the "metaphase rate" in normal and abnormal colonic mucosae in the rat. They estimated the mean cell cycle time in the lower four fifths of the crypt to be 32 h, in the normal descending colon.

In the present study several techniques have been employed to measure variation in proliferative activity from site to site along the length of the colon, and at different levels within the crypt in a single site in the descending colon of male Wistar rats.

Materials and Methods

Male Wistar rats aged three months and weighing between 400 and 500 g were used throughout. They were fed on standard rat cake (N.E. Farmers) and allowed water ad libitum. All the experiments were commenced at 9.00 a.m. in order to minimise any possible effects due to diurnal variation (Sigdestad et al., 1969; Al-Dewachi et al., 1976).

Distribution of Proliferative Activity within the Crypt

Five animals were given tritiated thymidine (^{3}HTdR) (Radiochemical Centre, Amersham, England), of specific activity of 5Ci/mMole, by intraperitoneal injection at a dosage of 0.5 μCi/g body weight. One hour after injection the animals were killed by cervical dislocation and the whole colon removed together with the caecum. The colon was opened along its length and emptied, then pinned to a cork board and fixed for six hours in Carnoy's fixative. After this initial fixation, transverse sections of the bowel were taken from the following sites:

(i) At a distance 30% of the way from anus to ileocaecal valve ("descending colon").
(ii) 60% of the distance from the anus ("transverse colon").
(iii) 90% of the distance from the anus ("ascending colon").
(iv) Distal third of caecum.

The sections were embedded in paraffin wax and serial 3 μm sections were processed for autoradiography (Al-Dewachi et al., 1974), the exposure period being 28 days.

For each animal, and in each of the four separate positions along the length of the bowel, the left sides of one hundred axially sectioned crypts were analysed. The cell positions of labelled nuclei and the cell positions of metaphase mitotic figures were recorded as well as individual crypt heights (Cairnie et al., 1965a). The criterion for the identification of labelled cells (Fig. 1) was five or more grains located over the nucleus (Wright, 1971). The data from these five animals

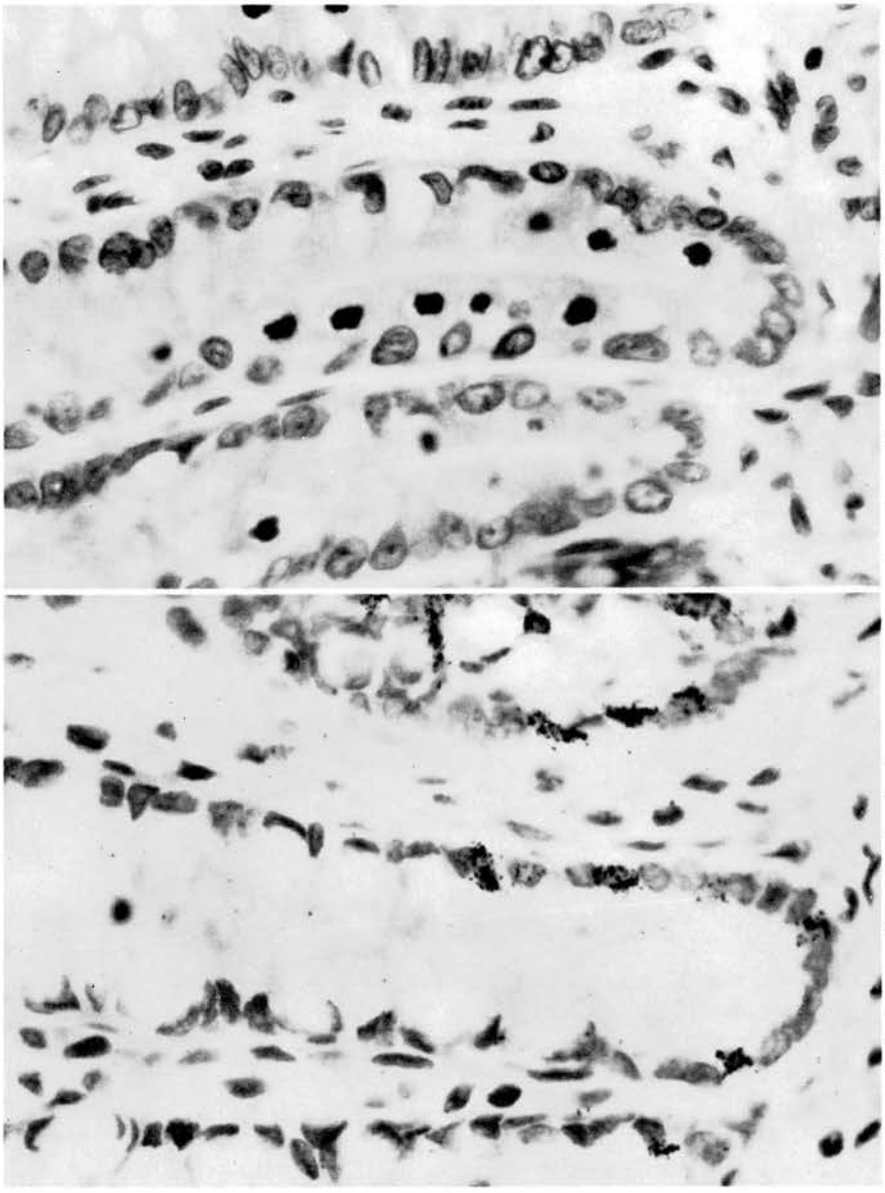

Fig. 1. Histological preparations showing the lower parts of crypts from descending colon. On the left there is an autoradiographic preparation showing labelling of enterocyte nuclei, on the right, vincristine arrested metaphases are seen. (Harris' haematoxylin × 750)

were combined and projected onto a standard crypt, the height of which was the mean crypt height (in cells) for each of the bowel positions. The modification by Wright et al. (1972), of the method of Cairnie and Bentley (1967), to compensate for variation in crypt height was used, to produce labelling and mitotic index distribution diagrams.

Stathmokinetic Study

Twelve animals were given vincristine sulphate ("Oncovin", Eli Lilly) by intraperitoneal injection at a dosage of 1 mg/kg body weight. The animals were killed serially at 15 min intervals thereafter.

Serial sections were prepared in the usual way on tissue taken from bowel position (i) "descending colon", and stained with Harris' haematoxylin. One hundred axially sectioned crypts were analysed and the cell positions of arrested metaphases (Fig. 1) were recorded, as well as the total crypt height. Adequacy of metaphase arrest was confirmed by the absence of any post metaphase mitotic figures in material taken 30 min and longer after the administration of vincristine. As before the data were projected onto a standard crypt. For the purpose of analysis this standard crypt was divided into cell position groups each consisting of four cells. The lowermost cell position group consisted of cell positions 1–4, the second group of cell positions 5–8, and so on up the crypt. For each of these cell position groups cumulative mitotic index was plotted against time.

From an analysis of two hundred crypt cross sections containing metaphases a correction factor was calculated to compensate for the overestimation of mitotic index due to migration of mitotic figures towards the centre of the crypt lumen (Tannock, 1967).

Continuous Labelling

Fourteen animals were given ^{3}HTdR at a dosage of 0.2 μCi/g body weight at five hourly intervals by the intraperitoneal route. The animals were killed serially at five hourly intervals, one hour after having received their last injection of label.

Autoradiographs were prepared as before from the descending colon following a two week exposure period. Again one hundred axially sectioned crypts were analysed; cell positions of labelled nuclei were recorded as well as total crypt height. The data were projected onto the standard crypt, and lines of cumulative labelling index against time were plotted for each of the cell position groups described in the stathmokinetic experiment.

Results

Distribution of Proliferative Activity within the Crypts

There are obvious morphological differences from site to site along the length of the rat colon. These mucosal changes are obvious even on a gross inspection of the opened bowel (Fig. 2), and the sites chosen for study are representative of these different mucosal patterns.

Particularly relevant, as far as a consideration of the distribution of proliferative activity within the crypt is concerned, is any variability in the mean height of crypts from site to site along the length of the colon. For each site in the bowel, and in each animal the mean crypt height (in cells) was calculated. The standard deviations were of the order of 3–5 cells. Table 1 shows the overall (inter-animal) means for each of the sites along the length of the bowel. The sizes of the "standard crypts", onto which all the data were projected are also shown. In the caecum and ascending colon the crypts are very considerably shorter than those in the transverse and descending colon.

Figure 3 shows the labelling index distribution curve for the descending colon, derived from the pooled data of the five pulse labelled animals killed 1 h after injection of ^{3}HTdR. Cell position in the crypt is plotted along the abscissa and labelling index (percent) along the ordinate. The shaded area indicates the 95% confidence limits for the points. In the lowermost cell positions mean values for the labelling index are only slightly lower than the peak labelling index of about 18% achieved in positions 5–10. Thereafter there is a steady decline in labelling index down to zero as the upper third of the crypt is reached. The mean crypt height at this site is forty two cells. Figure 4 shows

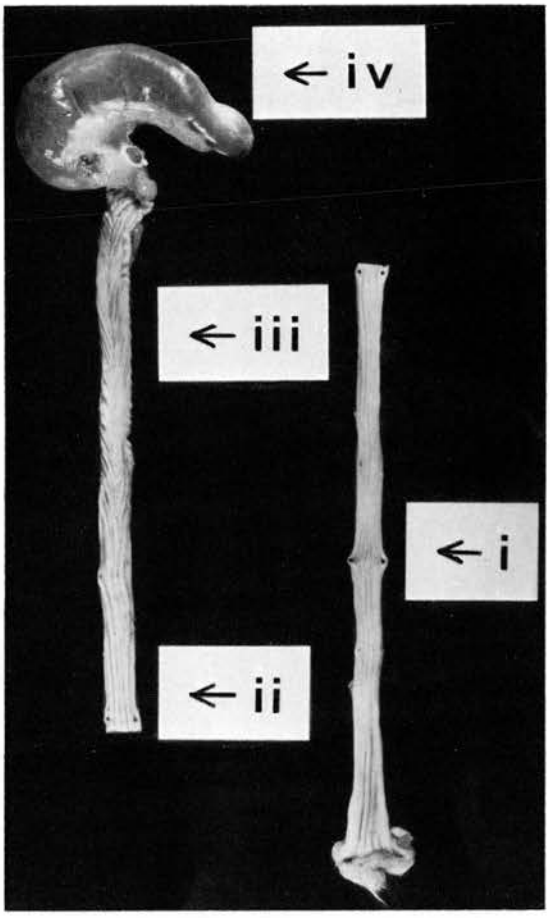

Fig. 2. Opened and fixed colon to show the variation in mucosal appearances, and the sites selected for microscopic examination. The colon has been divided at its midposition – caecum appears at upper left and anus below and on the right

Table 1. Mean crypt height (in cells) in the different sites in colon and caecum

		Mean crypt height (cells)	Size of standard crypt (cells)
(i)	Descending colon	42.1	42
(ii)	Transverse colon	44.0	44
(iii)	Ascending colon	30.4	30
(iv)	Caecum	30.8	31

the labelling index distribution curve for the transverse colon. Morphologically the colon at this site is very similar to that in the descending colon, and the mean crypt height is very similar also (44 cells as opposed to 42); however the labelling index distribution curve is strikingly different. Basally situated cell positions show low labelling indices, and labelling index steadily increases as the lower third of the crypt is traversed, to reach almost a plateau of 14–15%

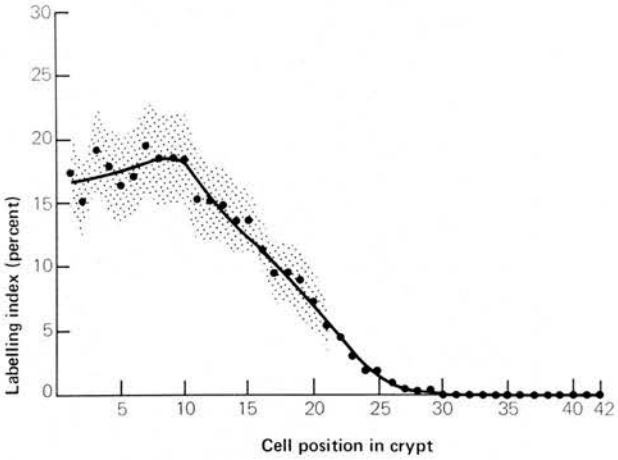

Fig. 3. Labelling index distribution curve for the descending colon (position i). The shaded area indicates the 95% confidence limits for the points

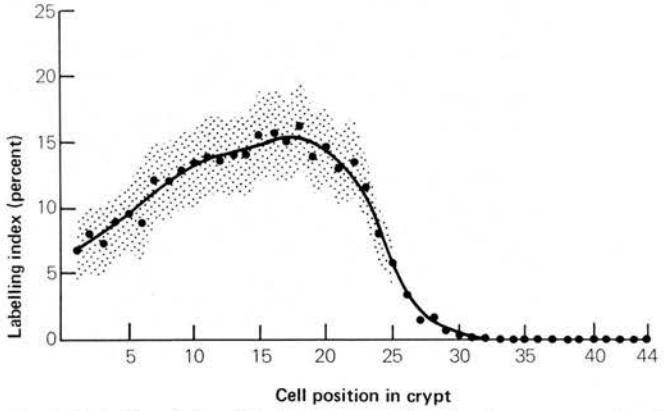

Fig. 4. Labelling index distribution curve for the transverse colon (position ii). The shaded area indicates the 95% confidence limits for the points

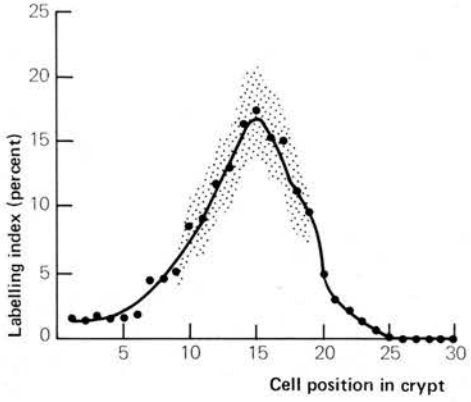

Fig. 5. Labelling index distribution curve for the ascending colon (position iii). The shaded area indicated the 95% confidence limits for the points

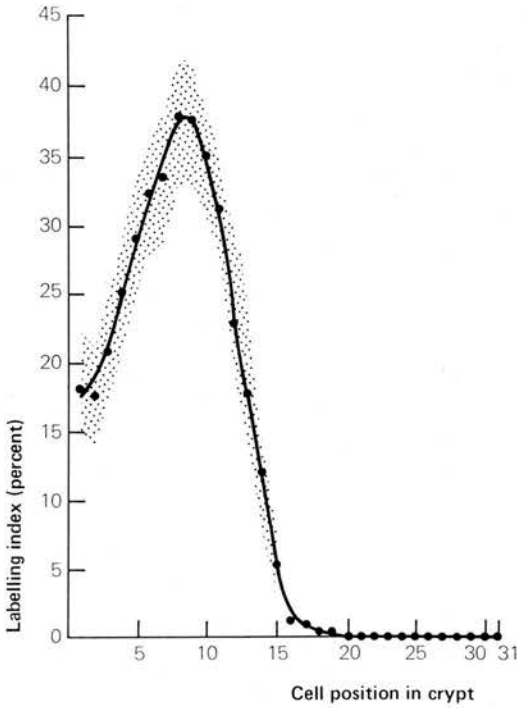

Fig. 6. Labelling index distribution curve for the caecum (position iv). The shaded area indicates the 95% confidence limits for the points

Table 2. Whole crypt mitotic index (I_m) and labelling index (I_s), with standard errors, at the various sites along the length of the bowel

		Size of standard crypt (cells)	Whole crypt I_m%	Whole crypt I_s%
(i)	Descending colon	42	0.51 (0.06)	7.52 (0.13)
(ii)	Transverse colon	44	0.32 (0.05)	6.93 (0.49)
(iii)	Ascending colon	30	0.34 (0.07)	5.50 (0.30)
(iv)	Caecum	31	0.61 (0.04)	12.33 (0.75)

in cell positions 10–20. Labelling indices fall abruptly to zero about two thirds of the way up the crypt.

In the ascending colon again quite a different distribution of labelling is apparent (Fig. 5). At this site basally situated cells show very low labelling indices (around 1%) and the labelling index then describes a sharp symmetrical peak in the middle third of the crypt. This reaches a maximum value of about 16% at cell position 15 of a thirty cell crypt.

In the caecum (Fig. 6) the labelling index is generally much higher, reaching peak values of about 38%, and again the picture emerges of basally situated cells showing lower labelling indices than the cell positions above them and with a rapid fall off of labelling indices through the middle portion of the crypt. The mean crypt height at this site is 31 cells.

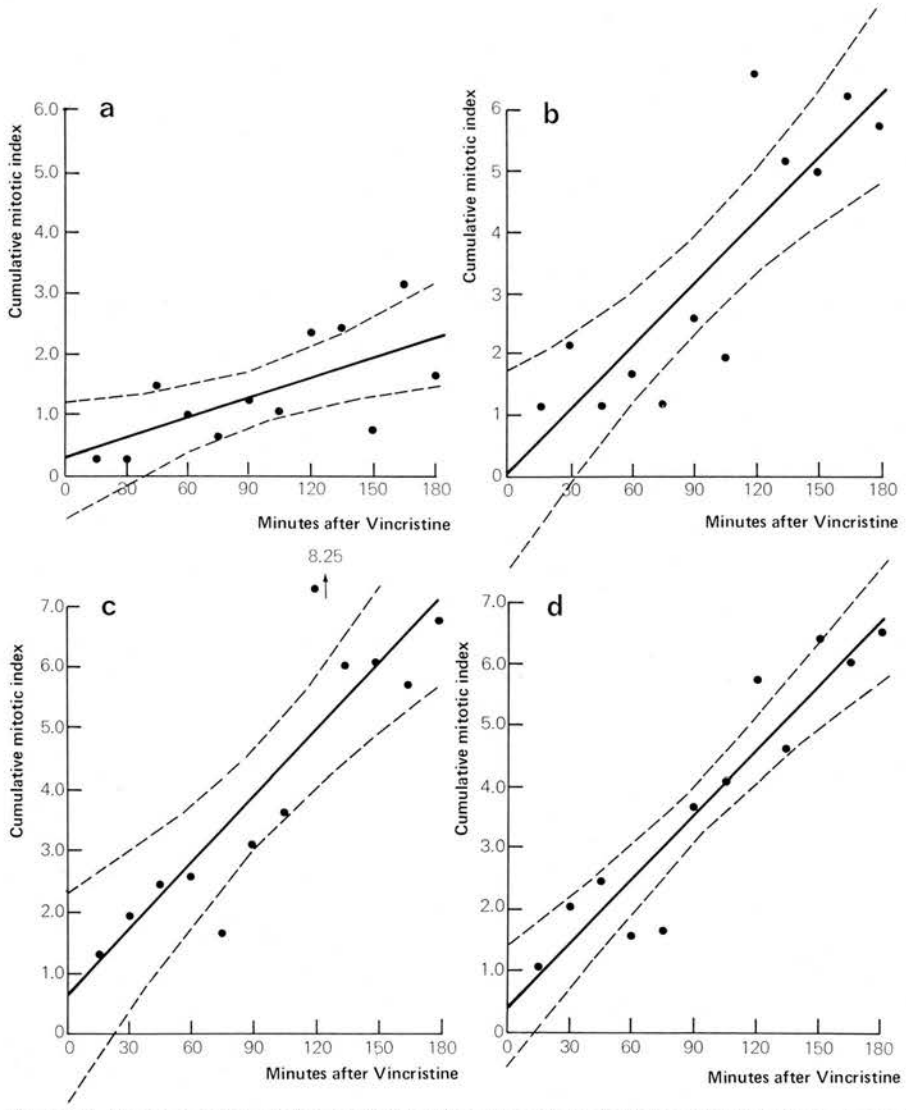

Fig. 7a–h. Cumulative mitotic index plotted against time after vincristine administration; descending colon. Curve **a** applies to cell position group 1–4; **b** applies to 5–8; **c** 9–12; **d** 13–16; **e** 17–20; **f** 21–24; **g** 25–28. Curve **h** is pooled, whole crypt data. The dotted lines indicated the 95% confidence limits for the line

Mitotic indices throughout the colon are low by comparison with the small intestine. Mitotic figures have been observed only in the lower part of the crypt. In Table 2 the crude "whole crypt" values for mitotic index are shown together with "whole crypt" values for labelling index for each of the positions along the bowel.

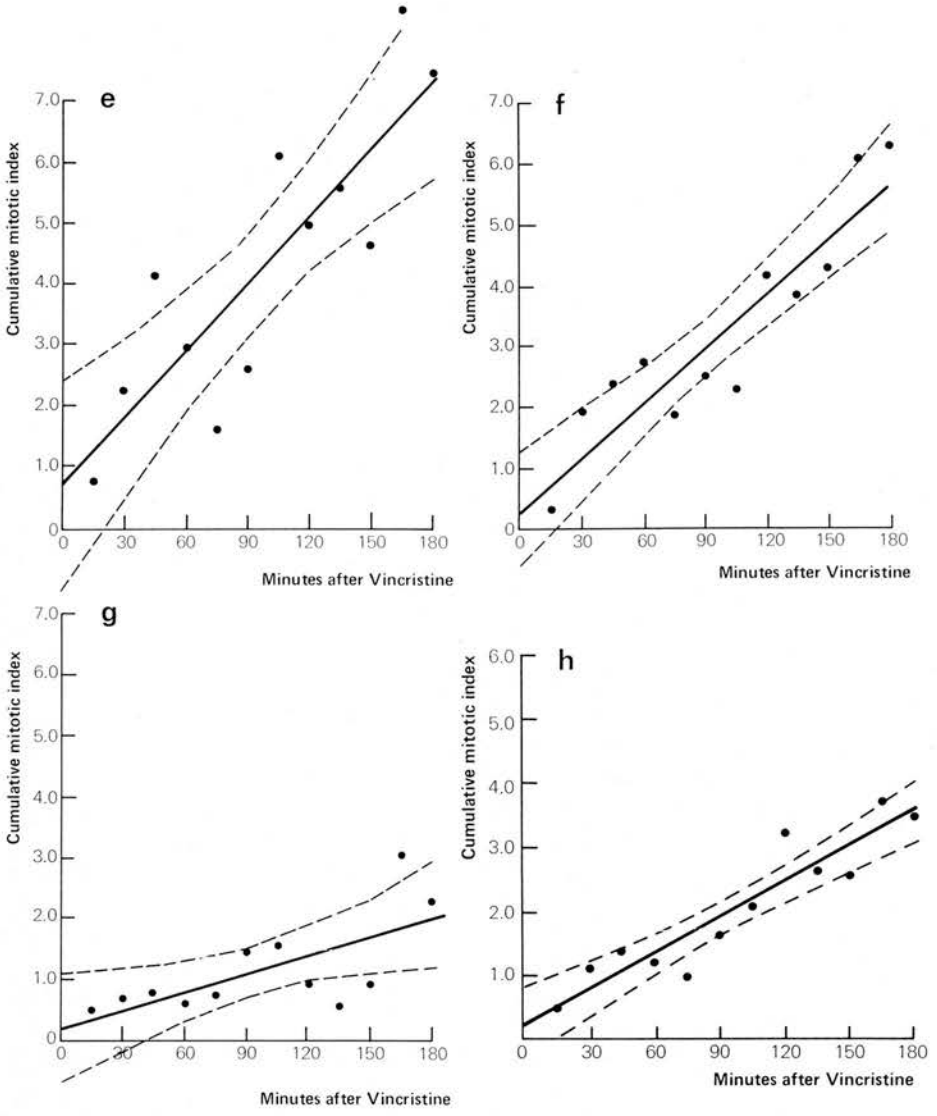

Fig. 7e–h

These preliminary observations show that as well as morphological mucosal differences, there exist significant differences in the kinetic organisation of the crypts from site to site along the length of the colon of the rat. These differences are masked by consideration of only the simple whole crypt mitotic index and labelling index data.

The succeeding observations apply to a single site in the descending colon (vide supra).

Table 3. Estimates of apparent cell cycle time ($T_{c(a)}$) in the various cell position groups in the descending colon, together with "whole crypt" $T_{c(a)}$

Cell position group	Apparent cell cycle time, h (confidence limits)
1–4	210 (113–1520)
5–8	71 (49–130)
9–12	64 (44–117)
13–16	67 (54–89)
17–20	65 (46–110)
21–24	82 (62–122)
25–28	233 (142–1816)
"Whole crypt"	130 (101–180)

Stathmokinetic Data

For each of the cell position groups described previously cumulative mitotic index was plotted against time. Figure 7a–g shows the fitted lines for each cell position group and Figure 7 (h) the pooled data for the whole crypt. Assuming an exponential age distribution in the proliferative compartment (Cairnie et al., 1965b; Tutton, 1973) estimates of apparent cell cycle time, $T_{c(a)}$ can be derived from the relationship

$$\log_2(1+I_m)=\frac{(t_m+t)}{T_{c(a)}}$$

where I_m is the mitotic index at time t, after vincristine and t_m is the duration of mitosis.

This estimate of apparent cell cycle time reglects however the overestimation of mitotic index caused by migration of mitotic figures into the lumen of the crypt (Tannock, 1967). Tannock's factor was found to have a mean value of 0.5 in two hundred crypt cross sections containing metaphases. In Table 3 are presented $T_{c(a)}$ estimates taking into account Tannock's factor with the appropriate 95% confidence limits. These confidence limits are very broad. In the basally situated cell position group $T_{c(a)}$ is prolonged by comparison with cell position groups higher up the crypt. Towards the top of the proliferative compartment we see a prolongation of $T_{c(a)}$: this is largely caused by the falling growth fraction, (I_p), which can be inferred from the shape of the labelling index distribution curve. As far as the whole crypt is concerned, a method has been suggested for estimating growth fraction from the form of the labelling index distribution curve (Cleaver, 1967). This is to determine the cell position at which the labelling index on the descending limb of the labelling index distribution curve is 50% of the maximum, and to express this as a fraction of the total crypt height. Therefore, in the descending colon, (Fig. 3)

$$\frac{\text{Cell position of 50\% of peak value}}{\text{Total height of crypt}}=\frac{19}{42}=0.45=I_p.$$

If an I_p of 0.45 is taken into account the actual cell cycle time becomes 58.5 h with confidence limits of 45 h and 81 h.

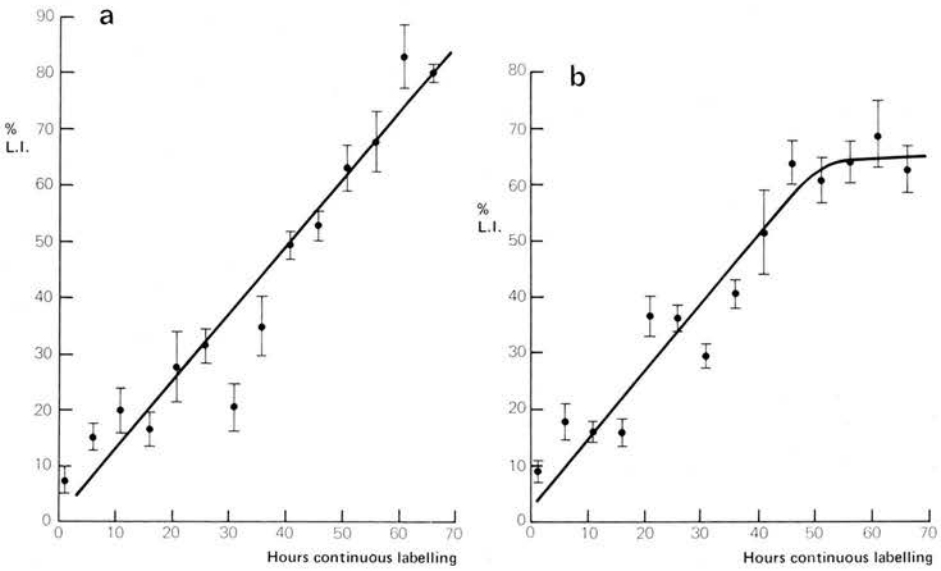

Fig. 8a and b. Cumulative labelling index plotted against duration of labelling (continuous labelling technique). Curve **a** applies to cell position grou 1–4; curve **b** to cell position group 13–16

Continuous Labelling Data

For each of the cell position groups previously described cumulative labelling index was plotted against time after the first injection of ^{3}HTdR. The duration of the experiment was 66 h, and in Figure 8 examples of the lines derived for two cell position groups are shown. Figure 8 (a) shows cell position group 1–4. In this position T_c is relatively prolonged, and as may be expected, no clear evidence of a plateau of labelling index is achieved over the experimental period. However, labelling index does reach very high levels (over 80%) and this implies a very high growth fraction. Figure 8 (b) shows the cumulative labelling index curve of the cell position group 13–16. After 40–50 h of continuous labelling a plateau value occurs. The time taken before this plateau is reached should be an indication of the duration of $(T_c - t_s)$. Thus a maximal estimate for T_c from this continuous labelling curve would be 60 h. The level of the plateau of labelling index should be related to I_p. In the absence of cell migration the plateau labelling value would be related to, but exceed, the growth fraction. However in this system there is cell migration and the I_p estimate of 50–60% derived from the continuous labelling curve should be interpreted with considerable caution: nevertheless I_p high up in the proliferative compartment would appear to be lower than that at the crypt base.

Discussion

From the labelling index distribution curves it is apparent that there are major differences in the distribution of proliferative activity within the crypt from

site to site along the length of the rat colon. These differences are not simply accounted for by variations in crypt height. These findings contrast with some published observations on the kinetic similarity of different sites in the small intestine (Clark, 1970; Altman and Enesco, 1967).

In the ascending colon of mice, Messier and Leblond, (1960), noted only rare labelled nuclei in the lower portion of the crypt: the present study shows that this is evident also in the ascending colon of the rat. However in the descending colon basal cells show only a suggestion of depression of labelling indices. Sawicki and Rowinski, (1970) studied sections of guinea pig colon "15 cm from colon–rectum junction": Kovacs and Potten (1973) do not state specifically the site in the colon from which their material was taken: Lipkin and Quastler (1962) used tissue from both ascending and descending colon, but do not state whether there were apparent any differences in morphology or labelling index distribution between these two sites. It is evident from the present study that such differences do exist, and that it is essential to state accurately the site from which material is studied before comparisons can be made.

In the descending colon analysis of stathmokinetic data shows that the basally situated cells have a prolonged apparent cell cycle time by comparison with those higher up the crypt. Analysis of stathmokinetic data presents several problems however. The first is a choice of age distribution. In the presence case we have chosen an exponential age distribution (Cairnie et al., 1965b; Tutton, 1973). A second problem is caused by the fall in growth fraction, the higher the cell position in the crypt. That such a fall in growth fraction occurs is manifest from the form of the labelling index distribution curve, but is impossible to quantitate from the present data. As far as the whole crypt is concerned however, I_p derived from the labelling index distribution curve permits the calculation of an actual cell cycle time of 58.5 h. This is considerably longer than the $T_{c(a)}$ of 32 h calculated by Tutton and Barkla, 1976, using a stathmokinetic technique in rats. Nor is this latter figure very dramatically altered by modifications due to growth fraction considerations and incorporation of Tannock's factor. Other cell cycle time estimates in the literature use animals other than rats, and are in general considerably shorter. This may be due to the use of labelling techniques. In mice Lipkin and Quastler (1962) estimated T_c at 16 h, while Potten et al. (1974) using a continuous labelling technique estimate T_c to be 18–20 h. Chang and Nadler (1975) calculated T_c at 23.2 h. Sawicki and Rowinski, (1970), working with guinea pigs found T_c to be approximately 20 h.

Our preliminary studies using the continuous labelling technique provide results which are in keeping with those derived from stathmokinetic studies. Basally situated cells appear to be cycling more slowly than those situated higher up the crypt. At the base of the crypt the growth fraction appears to be very high, whereas higher up in the proliferative compartment it certainly falls considerably. These observations confirm the suggestions of Messier and Leblond, (1960) and Lipkin and Quastler (1962). These slowly cycling basal cells may well represent the crypt stem cell population.

Acknowledgements. The financial support of the North of England Branch of the British Empire Cancer Campaign is gratefully acknowledged. We wish also to thank Mr. Bill Robinson who prepared the photographs and Miss Elizabeth Wark for typing the manuscript.

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Received August 29, 1977

David Appleton

Virchows Arch. B Cell Path. 32, 75–87 (1979)

Virchows Archiv B
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Cell Proliferation at Different Sites Along the Length of the Rat Colon

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Summary. This study has been undertaken in order to compare in detail cell proliferation in the mucosal crypts at several sites along the length of the large bowel of the rat. The techniques which have been used include simple morphometry, calculation of mitotic and tritiated thymidine labelling indices, metaphase arrest with vincristine, and the fraction of labelled mitoses method.

Major differences exist in the size and shape of the crypts at different sites. In particular the distribution of the proliferating cells within the crypt varies. Mean cell cycle time ranges from 58 h in the descending colon to 25 h in the caecum; this variation appears to be brought about largely by changes in the duration of G_1, the other phase durations remaining relatively constant. There is also variation in cell cycle time and growth fraction at different levels within the crypt; throughout the bowel cells appear to cycle more slowly at the bottom of the crypt, but changes in growth fraction do not display a similarly consistent pattern.

Clearly the organisation of cell proliferation in the normal rat colon is very complex, and strict definition of anatomical location is required in any study of cell proliferation whether in normal or in diseased animals.

Key words: Colon – Kinetics – Thymidine – Vincristine.

Introduction

The morphology of the normal colonic mucosa in the rat changes from one site to another along the length of the organ. In a previous communication (Sunter, Wright and Appleton, 1978) we described these differences in terms of crypt length and the distribution of mitotic and tritiated thymidine (^{3}HTdR)

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0340-6075/79/0032/0075/$02.60

labelled cells. These changes in the pattern of the normal mucosa are of interest, especially in view of the fact that the different histological types of colonic tumor occurring in the dimethylhydrazine (DMH) model of intestinal carcinogenesis show different distribution patterns (Ward, 1974; Reddy, Narisawa and Weisburger, 1976; Sunter, Appleton, Wright and Watson, 1978). These tumor distributions mirror the changing pattern of the normal colonic mucosa and we have suggested that the basic cytokinetic organisation of the normal crypt might be one of the factors that determine the outcome of the carcinogenic stimulus (Sunter et al., 1978).

This study has been undertaken to define as precisely as possible the proliferative characteristics of the normal mucosa at several sites within the large bowel of the rat. Morphometric techniques have been employed in conjunction with pulse labelling with ^{3}HTdR, metaphase arrest experiments with vincristine, and the fraction of labelled mitoses (FLM) method.

Materials and Methods

Randomly bred virgin female albino Wistar Porton rats were used throughout. The animals were between 12 and 14 weeks old when the experiments were carried out, and weighed between 225 and 275 g. They were fed on standard rat cake (N E Farmers, Aberdeen) and allowed tap water ad libitum.

Mitotic and Labelling Indices

Four animals were given tritiated thymidine (Radiochemical Centre, Amersham, England) of specific activity 5 Ci/mMole by intraperitoneal injection at 1400 h, the dosage being 0.5 μCi/g body weight. One hour after the injection each animal was killed by cervical dislocation. The whole colon was removed in continuity with the caecum and opened longitudinally, then pinned mucosal surface uppermost to a cork board. The entire specimen was fixed in Carnoy's fluid for 6 h, and following this fixation transverse sections of the full thickness of the bowel were taken from the following sites:

(i) Descending colon – 30% of the distance measured from the anus to the ileocaecal valve
(ii) Transverse colon – 60% of that distance
(iii) Ascending colon – 90% of that distance
(iv) Caecum – the junction of the distal and middle thirds of the caecum.

These blocks were transferred to 2-ethoxy-ethanol, and then processed through to paraffin wax. Serial transverse histological sections 3 μm thick were cut and autoradiographs were prepared in the usual way using a dipping technique. The exposure period was four weeks.

For each sample the "left sides" of one hundred perfect axial sections of crypts were analysed. The total length of the crypt in cells (the crypt column) was recorded, together with the cell positions of mitotic figures and of labelled nuclei, the criterion for identification of labelling being five or more autoradiographic grains directly over the nucleus. The data from the four animals were combined, and using the computer method of Cairnie and Bentley (1967) as modified by Wright, Morley and Appleton (1972) labelling and mitotic index distribution diagrams were produced for each of the four separate sites within the bowel. The length of each of these "standard crypts" was the mean length of all the crypts counted at any particular site both in this group and in the vincristine group.

Vincristine Study

Eleven animals were given vincristine sulphate ("Oncovin", Eli Lilly) by intraperitoneal injection at 0900 h in a dosage of 1 mg/kg body weight. Animals were killed at intervals of 15 min thereafter,

the first at 15 min after the vincristine and the last at 165 min. The colons were removed and fixed as described above, and 3 μm thick histological sections were prepared from each of the selected sites. Staining was with Harris's haematoxylin. In each sample the "left sides" of one hundred perfect axial sections of crypts were analysed, the positions of arrested metaphases and the total crypt length being recorded. In material taken 30 min and longer after the administration of vincristine there were no post-metaphase mitotic figures, thus confirming the adequacy of metaphase arrest. As before the data were expressed in terms of a standard crypt.

From four animals in this group transverse histological sections of crypts were prepared at each of the four sites in the colon. From analysis of fifty crypts in each of the four samples mean values of the number of cells around the crypt circumference were obtained (the crypt column count). From analysis of the same number of sections containing metaphases a correction factor was calculated to compensate for the overestimation of mitotic index caused by movement of mitotic figures towards the lumen of the crypt (Tannock, 1967).

FLM Study

Thirty-seven animals were given ^{3}HTdR by intraperitoneal injection at a dosage of 0.5 μCi/g body weight at 0900 h. Animals were killed at hourly intervals, commencing 1 h after injection, for the first 20 h, and thereafter at two hourly intervals up to 54 h. Autoradiographs were prepared from each of the defined sites as described above. For analysis, the crypt was divided into cell position groups, each consisting of four cell positions. The lower group consisted of cell positions 1 to 4, the second group positions 5 to 8 and so on up the crypt. In each sample a minimum of twenty mitotic figures was scored for each cell position group and the proportion of mitotic figures which were labelled was determined. In this way for each of the separate cell position groups an FLM curve was constructed. In the ascending colon (site iii) it was impracticable to analyse the crypts in this way, partly because of the paucity of mitotic figures in the lower third of the crypt, and partly because of the great variability in the length of individual crypts: and so these crypts were simply divided into upper and lower halves. An FLM curve for the "whole crypt" was constructed from the means of the values obtained in each of the component cell position groups for each site along the length of the bowel. All the FLM curves were analysed by the method of Gilbert (1972).

Results

Table 1 summarises the whole crypt data derived from morphometry and studies of mitotic index (I_m) and the information obtained from the metaphase arrest

Table 1. Kinetic data for the whole crypt

Variable		Derivation	(i)	(ii)	(iii)	(iv)
a	Crypt length (cells)	Observed	41.8	43.0	33.2	32.8
b	Crypt column count	Observed	17.6	17.1	19.0	23.2
c	Crypt cell population	$a \times b$	735	735	630	760
d	Observed mitotic index	Observed	0.0056	0.0048	0.0059	0.0055
e	Tannock's factor	Observed	0.63	0.63	0.63	0.56
f	Corrected mitotic index	$d \times e$	0.0035	0.0030	0.0037	0.0031
g	Birth rate (cells/cell/h)	note 1	0.010	0.013	0.012	0.015
h	Cumulative I_m (cells/col)	$a \times f$	0.15	0.13	0.12	0.10
i	Cumulative K_B (cells/col/h)	note 2	0.42	0.51	0.34	0.43
j	Mitotic duration (h)	h/i	0.36	0.25	0.35	0.23
k	Crypt cell production rate (cells/h)	$b \times i$	7.3	8.7	6.5	9.9

Note 1. From the slope of the line of metaphase accumulation following administration of vincristine and assuming a rectangular age distribution

Note 2. Assuming an exponential age distribution at individual cell positions

experiment with vincristine. The mean crypt lengths in the descending colon and transverse colon are virtually the same, while in the ascending colon and caecum the mean value is considerably smaller. The amount of variation in the length of the crypts at a given site within the bowel in any particular animal also changes. In the ascending colon, with its complicated pattern of mucosal folds, this variability is greatest with standard deviations of the distribution of crypt lengths in individual animals of the order of 4 cells; in the other selected sites the standard deviations are between 2 and 2.5 cells. The crypt column counts vary in the opposite manner to crypt length, so that the shortest crypts have the greatest circumference and vice versa. The product of the crypt length and the column count has been evaluated in order to give an estimate of the total cell population of the crypts. In the ascending colon the crypt cell population would appear to be slightly smaller than that at the other sites, which show very similar population sizes.

The means for the observed mitotic indices in the crypt as a whole show small differences; these observed values have been multiplied by a correction factor to compensate for overestimation of I_m caused by counting axial sections of crypts (Tannock, 1967). The correction factors are given, along with the corrected I_m values. All subsequent calculations have taken this geometric artefact into consideration.

For each site in the bowel, whole crypt I_m has been plotted against time after administration of vincristine. In this consideration of the crypt as a whole we have assumed a rectangular age distribution, and the line has been fitted by least squares (Fig. 1). From the slopes of the lines estimates of birth rate (K_B) are obtained. Birth rate is greatest in the caecum and least in the descending colon.

An estimate of cumulative I_m (mitoses/crypt column) can be arrived at by multiplying whole crypt I_m, which has been expressed as a proportion, by the mean crypt length. These values are given in Table 1. From the distribution within the crypt of metaphases arrested following vincristine administration we can produce graphs of cumulative mitotic index at each separate cell position within the crypt, and from these graphs estimates of K_B at individual cell positions can be arrived at (Rodriguez et al., 1979). In dealing with these cell positions an exponential age distribution has been assumed. Figure 2 shows these individual birth rates plotted cumulatively. The cumulative birth rate at the top of the crypt gives an indication of the number of new cells produced per crypt column per hour. The cell production rate for the crypt is evaluated from the product of the column count and the cumulative K_B at the top of the crypt. A relatively accurate estimate of mitotic duration can be obtained from these data also (Rodriguez et al., 1979). All these figures are summarised in Table 1.

The curves fitted to the whole crypt FLM data by the Gilbert program are shown in Fig. 3. The cell cycle phase durations calculated from these curves are tabulated (Table 2). Both t_s and t_{G2} appear to be relatively constant at around 9 h and 2 h respectively, and the considerable differences in the cell cycle time (T_c) from one site in the bowel to another seem to arise because of variation in the duration of the G_1 phase. The estimate of the coefficient

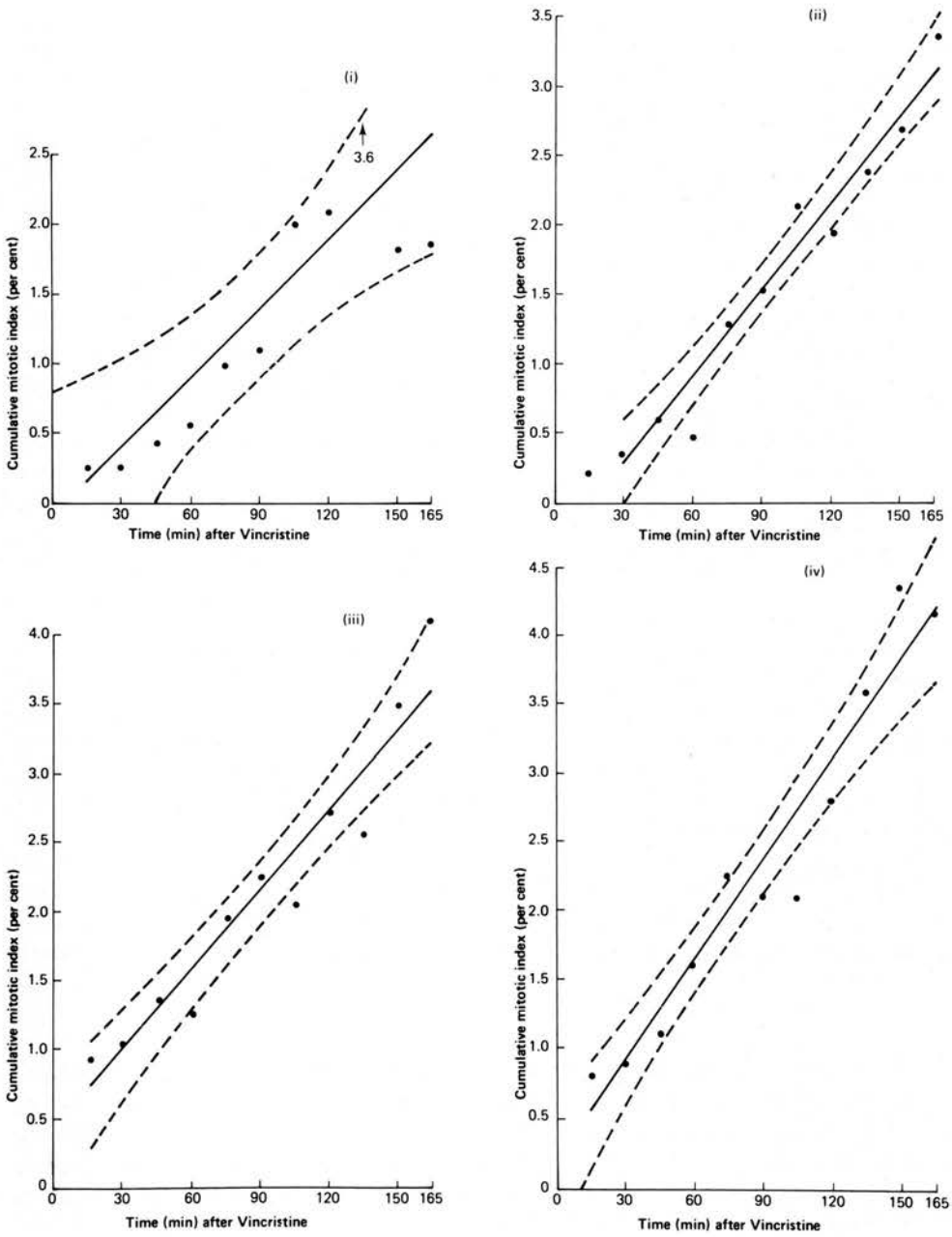


Fig. 1. Mitotic accumulation data for the whole crypt following administration of vincristine. The broken lines indicate 95% confidence limits for the fitted lines. **(i)**, **(ii)**, **(iii)** and **(iv)** correspond to the sites described in the text

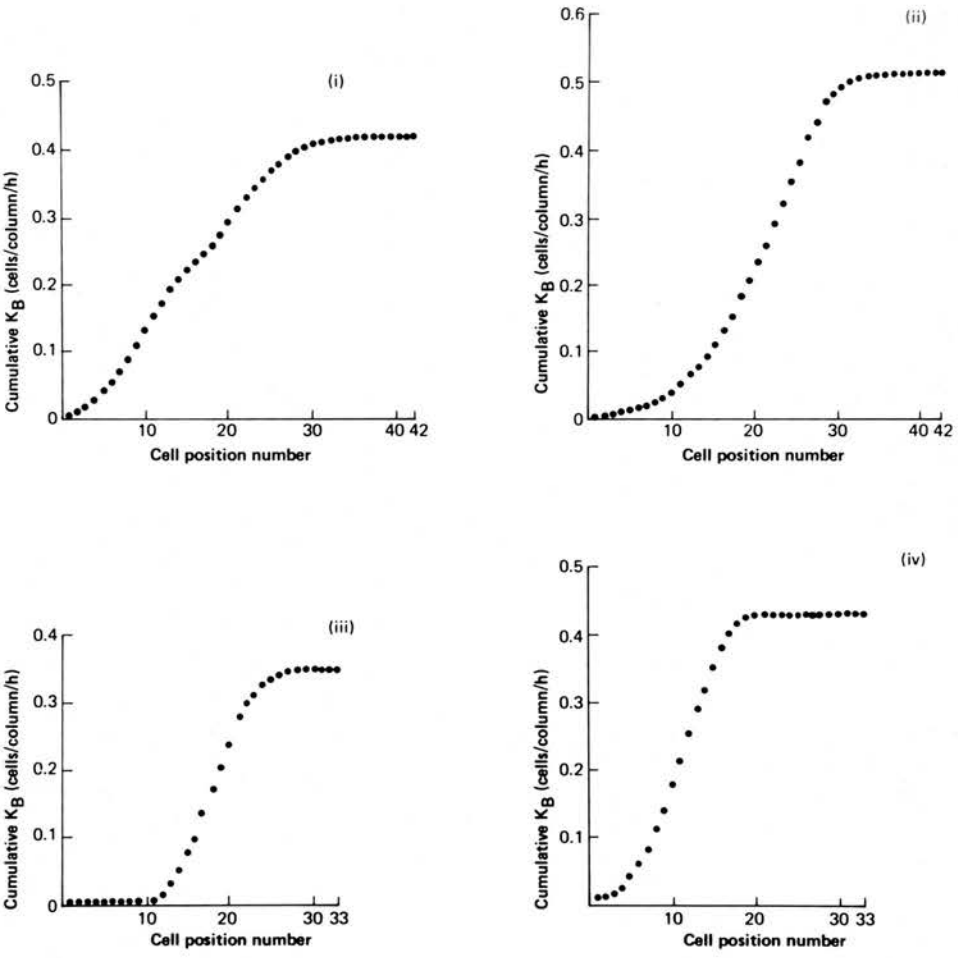


Fig. 2. Cumulative birth rate plotted against cell position in the crypt. **(i)**, **(ii)**, **(iii)** and **(iv)** correspond to the sites described in the text

of variation of T_c provided by the Gilbert program is also shown. A theoretical labelling index is calculable from the cell cycle phase durations. We have assumed a rectangular age distribution for the whole crypt, and hence the theoretical labelling index is derived from the quotient t_s/T_c. These figures are given in Table 2. We have shown however that failure to take into account the variability of T_c can result in either overestimation or underestimation of the theoretical labelling index and have described a simple method of calculating more accurate estimates (Appleton and Sunter, 1979). The correction factors calculated for the present data are given to emphasise their magnitude and the corrected theoretical labelling indices are shown as well as the mean observed labelling indices from the group of ^{3}HTdR pulse-labelled animals. From the ratio of observed to theoretical labelling index estimates of growth fraction (I_p) are

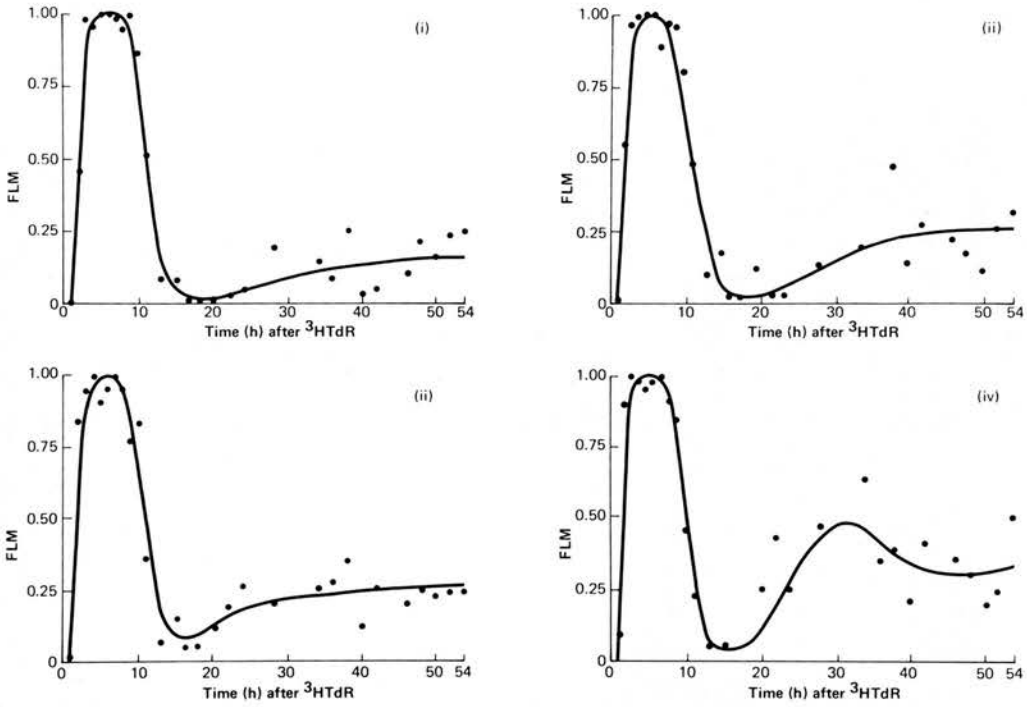


Fig. 3. Fraction of labelled mitoses curves, fitted by the method of Gilbert (1972) to whole crypt data, **(i)**, **(ii)**, **(iii)** and **(iv)** correspond to the sites described in the text

Table 2. Kinetic data for the whole crypt (contd.)

Variable		Derivation	(i)	(ii)	(iii)	(iv)
l	t_{G1} (h)	Note 1	46.6	30.7	24.6	15.2
m	t_s (h)	Note 1	9.0	9.1	8.8	8.5
n	t_{G2} (h)	Note 1	2.0	2.0	1.6	1.5
o	T_c (h)	Note 1	57.9	42.0	35.3	25.4
p	Coefficient of variation of T_c		0.52	0.38	0.54	0.26
q	Theoretical labelling index	m/o	0.16	0.22	0.25	0.33
r	Correction factor	Note 2	1.33	1.11	1.27	1.08
s	Corrected theoretical labelling index	$q \times r$	0.21	0.24	0.32	0.36
t	Observed labelling index	Observed	0.071	0.072	0.073	0.109
u	Growth fraction	t/s	0.34	0.30	0.23	0.30
v	Total number of proliferating cells	$u \times c$	250	220	145	230
w	Birth rate (FLM study) cells/cell/h	u/o	0.006	0.007	0.007	0.012
x	Half maximum position	Note 3	14	26	20	13
y	Cut-off position	Note 4	11	17	16	10

Note 1. Values derived from FLM analysed by the Gilbert method (1972)
Note 2. To compensate for inaccuracies due to variability in T_c (Appleton and Sunter, 1979)
Note 3. The cell position at which I_s diminishes to half its maximum value (Cleaver, 1967)
Note 4. The average position at which cells embark on their final proliferative cycle (Appleton, Sunter and Watson, 1979)

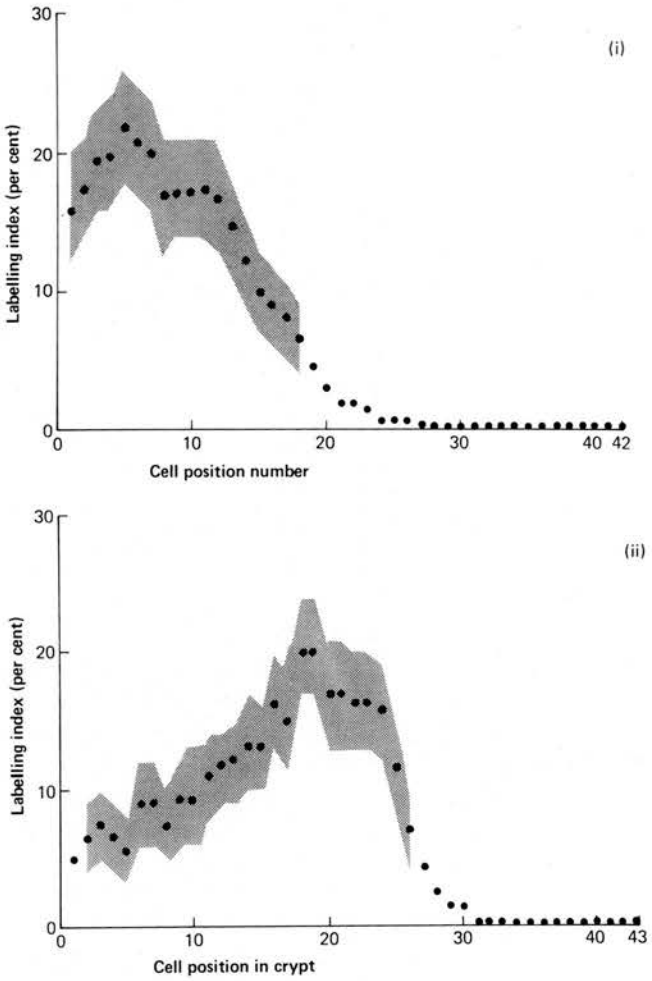
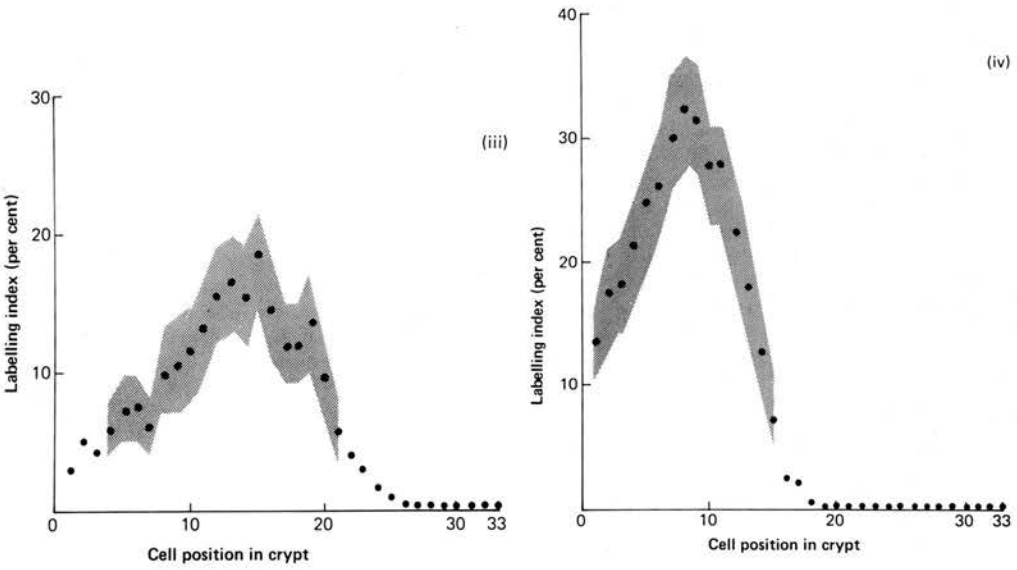


Fig. 4i–iv. Labelling index distribution in the crypt: **(i)** the descending colon, **(ii)** the transverse colon **(iii)** the ascending colon and **(iv)** the caecum. The shaded areas indicate the 95% confidence limits for the points

obtained. The product of I_p and the total crypt cell population gives an estimate of the total number of proliferative cells per crypt, and a further estimate of K_B is obtained by dividing I_p by T_c. These estimates of K_B obtained purely from the ^{3}HTdR labelling studies are smaller than those obtained from the vincristine data, but show a similar pattern with the largest value in the caecum and the smallest in the descending colon. These K_B estimates and the other variables referred to are all shown in Table 2.

The labelling index distribution diagrams for each of the four sites along the length of the bowel are shown in Fig. 4. In the descending colon labelling activity is confined to the lower half of the crypt, with maximal I_s values



of about 20% occurring near the crypt bottom, and only a slight depression of I_s actually at the bottom of the crypt. In the transverse and ascending colon (sites ii and iii) this depression of basal I_s is much more conspicuous and peak labelling of the order of 20% is found near the middle of the crypt, after which there is a rapid decline. In the caecum labelling indices are much higher, but the overall pattern of proliferating cells confined to the lower part of the crypt, and a lower I_s at the crypt base than is found higher up, is maintained. From the study of the I_s distribution diagrams it is clear that the disposition of proliferating cells within the crypt varies from one site to another along the length of the colon. Cleaver (1967) suggested that an indication of the absolute size of the proliferative compartment might be gained from the position at which I_s within the crypt fell to half its maximum value, the half maximum position. These values are given in Table 2, along with the estimates of the cut-off position (the average cell position at which cells enter their final cycle) calculated from the stathmokinetic data (Cairnie, Lamerton and Steel, 1965; Appleton, Sunter and Watson, 1979).

The estimates of cell cycle times derived from analysis of the FLM curves for each of the separate four-cell position groups are given in Table 3. In the descending colon, transverse colon, and caecum, T_c appears to be relatively prolonged at the base of the crypt when compared with the cell position groups situated higher up the crypt. In the ascending colon T_c is slightly greater in the lower half of the crypt than in the upper. Estimates of I_p, derived from the ratio of observed I_s to theoretical I_s, calculated from the cell cycle parameters obtained from the FLM data assuming an exponential age distribution and compensating for variability in T_c (Appleton and Sunter, 1979), are shown in Table 4.

Table 3. Cell cycle times (h) at different sites within the crypts, calculated from FLM curves analysed by the method of Gilbert (1972)

Cell position group	1–4	5–8	9–12	13–16	17–20	21–24
Descending colon (i)	68.4	61.0	64.9	45.9	53.5	71.5
Transverse colon (ii)	70.7	64.4	39.8	38.5	38.7	37.4
Ascending colon (iii)			39.7			32.1
Caecum (iv)	34.6	25.4	23.4	24.1	–	–

Table 4. Growth fraction estimates calculated assuming exponential age distributions and corrected for variation in cell cycle time

Cell position group	1–4	5–8	9–12	13–16	17–20	21–24
Descending colon (i)	1.15	1.20	0.95	0.58	0.36	0.10
Transverse colon (ii)	0.29	0.61	0.45	0.59	0.88	0.87
Ascending colon (iii)			0.36			0.28
Caecum (iv)	0.65	0.87	0.88	0.35	–	–

Discussion

This study has confirmed our previous finding (Sunter et al., 1978). That the size and shape of the mucosal crypts vary considerably from one site to another along the length of the rat colon. The length of the crypts in the descending colon proved to be the same as that described in our previous report, 42 cells. This value is similar to that observed by Tutton and Barkla (1976), which was 38.2 cells. Rijke et al. (1979) observed a value of only 32.8 cells, and ascribed this shortness to the relative youth of their animals. Their estimate of crypt circumference, 18.9 cells, was very similar to our 17.6 cells, and about half that of Tutton and Barkla (1976). These latter authors estimated total crypt population in the descending colon at approximately 1,300 cells; Rijke and his coworkers' estimate was 623 cells, and our estimate from the present study 735 cells.

As far as the rest of the large bowel is concerned the results show a very similar pattern to our previous observations (Sunter et al., 1978). In the transverse colon the crypt length and circumference are similar to those observed in the descending colon. In the ascending colon and caecum the crypts are about 10 cells shorter than this, but apparently slightly longer than the corresponding values we observed in male animals. Changes in the circumference of crypts at different sites tend to compensate for changes in length, so that the total crypt population in fact varies relatively little from one site to another: in the ascending colon it is smallest at 630 cells, and greatest in the caecum at 760 cells.

Overall mitotic indices corrected for the geometric artefact described by Tannock (1967) range from 0.3% to 0.4% at different sites in the colon, and the corresponding labelling indices range between 7.1% and 10.9%. These figures

are very similar to those we described previously (Sunter et al., 1978). The distributions of labelled cells are also very similar, and such differences as do exist are possibly explicable on the basis of diurnal variation, since relatively big changes have been described (Hamilton, 1979). In the descending colon the distribution of labelled cells is very similar to that described by Rijke and coworkers (1979) and by Wiebecke et al. (1973), although our peak labelling indices are slightly higher; but these latter authors did not observe the basal depression of I_s, possibly because they did not divide the crypt into small enough sections. The quite different distribution of labelled cells in the ascending colon is in keeping with the description of Messier and Leblond (1960). It seems clear that in the colon the proliferating cells are confined to the lower part of the crypt as in the small intestine. There has been some debate as to how the size of this proliferative compartment should be measured. We agree with Steel (1977) that a useful function of proliferative compartment size is that cell position at which cells will enter their final cycle, and a method has been described which is applicable when I_p is less than unity within the proliferative compartment (Appleton et al., 1979). The site of these cut-off positions varies considerably: in the descending colon it is position 11, while in the transverse colon, virtually identical in crypt size, it is position 17, in the caecum position 10, and in the ascending colon position 16.

Analysis of the FLM data for the whole crypt has produced estimates of the durations of the major cell cycle phases. In the descending colon mean T_c is 58 h, and there is a gradual shortening of T_c in the more proximal sites, with the smallest value, 25 h, seen in the caecum. This change would appear to be due largely to a shortening in the duration of G_1 since the other phase durations remain fairly constant. Rijke and his colleagues (1979), using the FLM technique, found a T_c of 50.5 h in the descending colon as a whole, and a t_s of 7.6 h; both these estimates are only slightly shorter than ours. In our previous study (Sunter et al., 1978) T_c was calculated at 58 h from stathmokinetic data, correction having been made for the size of the growth fraction. Tutton and Barkla (1976) used the stathmokinetic technique, and estimated an "apparent cell cycle time" as 32 h in the descending colon.

The FLM curves have been analysed by cell position groups within the crypt (see Materials and Methods). Each group consisted of four cell positions, save in the ascending colon where a paucity of mitotic figures associated with great variability in the lengths of individual crypts made it impracticable to analyse the material in this way; consequently data were collected for upper and lower halves of the crypt. All sites in the bowel share the common pattern that cells in the lowest part of the crypt have relatively longer cell cycle times, while cells higher up the proliferative compartment are cycling more rapidly. This finding is in keeping with previous observations on the descending colon of the rat (Sunter et al., 1978; Rijke et al., 1979) but contrasts with the situation in the colon of normal mice (Rodriguez et al., 1979; Sunter et al., 1979). These slowly cycling cells at the bottom of the crypt may well represent a population of stem cells. The growth fraction shows important variations; it is highest at 0.34 in the descending colon, while in the short crypts of the ascending colon I_p is only 0.23; transverse colon and caecum show intermediate values.

At different levels within the crypts it shows some interesting variations which need an explanation. In the descending colon it is highest at the bottom of the crypt, where it is unity, and it steadily declines with rising cell position. This is in keeping with previous findings (Sunter et al., 1978; Rijke et al., 1979), and is readily explicable on the basis of increasing cell differentiation. In the transverse colon, however, relatively low values of I_p are seen in the lower parts of the crypt, with the maximum values (approaching unity), occurring in the mid portion of the crypt, where the labelling index is highest. For the ascending colon the data are poor, but the form of the labelling index distribution curve is such that a similar situation is likely. Is there then a separate subpopulation of non-proliferative or differentiated cells at the bottom of the crypts of the proximal half of the colon? Ward (1974) commented on the large population of mucous secreting goblet cells at the bottom of the crypts in the proximal rat colon, and his observations accord with ours. These cells may well represent such a population of differentiated non-cycling cells, whose presence in no way militates against the coexistence of a stem cell population in the same zone.

We have calculated the total crypt cell-production rate from combined stathmokinetic and morphometric data. Crypts in the descending colon produce 7.3 new cells/h while in the transverse and ascending colon the figures are 8.7 cells/h and 6.5 cells/h respectively; in the caecum 9.9 cells/h are produced. These figures are remarkably similar when one considers the many ways in which the details of kinetic organisation differ from one site to another. The organisational differences must surely reflect differences in function: and in turn may determine the way in which the mucosa is programmed to respond to damaging influences.

Acknowledgements. This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We wish to thank Miss E. Wark who typed the manuscript, and Mrs. E. Wallace who provided invaluable technical assistance and Mr. R. Joyce who prepared the illustrations.

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Received July 23, 1979

David Appleton

J. Anat. (1986), **149**, pp. 177–188
With 4 figures
Printed in Great Britain

A comparison of crypt-cell proliferation in rat colonic mucosa *in vivo* and *in vitro*

20

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(*Accepted* 4 *February* 1986)

INTRODUCTION

Many studies have been undertaken to investigate cell proliferation of both normal and neoplastic tissue in the hope that an understanding of any fundamental differences in the kinetics would provide information on the process of tumour development and help in designing strategies for treatment. Studies *in vivo* have been greatly facilitated by the development of reliable animal models as the *in vivo* data available for man is both limited and unsatisfactory. The development of a system for the *in vitro* culture of tissue solved many of the problems encountered in the intact organism and consequently was employed by several groups.

It has been common practice to study the kinetics of tissue that has been cultured for relatively short periods of time (Denekamp & Kallman, 1973; Deschner, Lewis & Lipkin, 1963; Fabrikant, Wisseman & Vitak, 1969; Hendrickson & Skypeck, 1963; Johnson & Bond, 1961; Lieb & Lisco, 1966; Meyer & Connor, 1977; Wolberg & Brown, 1962). But it is now known that complex changes in proliferation can occur early in culture and that mitosis and DNA synthesis are initially depressed (Simnett & Fisher, 1973). Pritchett (1982), and Pritchett *et al.* (1981) investigated the labelling and mitotic indices in colonic tumours and in normal colonic mucosa over a period of 24 hours and, having discovered that values settled only after 18 hours in culture, stressed the importance of selecting the optimum time for performing kinetic experiments. Attempts have been made, mainly in tumour material, to investigate the reliability of kinetic data obtained in short-term *in vitro* studies by comparison with data from *in vivo* sources, and favourable results have been reported (Denekamp & Kallman, 1973; Fabrikant, *et al.* 1969; Hendrickson & Skypeck, 1963; Meyer & Connor, 1977; Rajewsky, 1965). However, the limitations of short term culture make an extension of the culture period highly desirable.

A number of workers have described systems designed for the long-term preservation of colonic mucosa in culture. Schiff & Moore (1980) were able to maintain adult rat colonic mucosa in stationary culture, using fibrin foam as the support, for 21 days. Defries & Franks (1977) successfully cultured viable mouse colon on stainless steel grids for up to 3 weeks. Both rodent (Reiss & Williams, 1978, 1979; Shamsuddin *et al.* 1978) and human colonic mucosa (Autrup *et al.* 1978; Autrup, 1980) have been maintained for extended periods using a rocking technique with the addition of steroid hormones. The culture system used in the present study was developed initially by Hodges & Melcher (1976) but was further modified by Senior, Sunter, Appleton & Watson (1984) and Senior *et al.* (1982) for the long-term culture of both human and rodent colonic mucosa. The maintenance of the cultured tissue

is not only prolonged but good structural and functional integrity is also achieved. The effect of drugs or radioactive isotopes on normal human mucosa and colorectal tumour can thus be studied when, for ethical reasons, it is not possible to do so *in vivo*.

We are using the long-term culture system for a study of putative trophic factors in the absence of some of the complex influences which occur *in vivo*. However, culture itself may change normal cell proliferation and this must be taken into consideration. Indeed does cell proliferation in the *in vitro* system bear a constant relationship to that *in vivo*?

In this paper we present an investigation to characterise the culture system by a comparison of the proliferative parameters *in vivo* with those *in vitro*. In the light of the results of the studies made by Pritchett (1982) and Pritchett *et al.* (1981) experiments were performed in culture at a time when we expected kinetic stability. Any changes in proliferation discovered between the animal tissue *in vivo* and *in vitro* may provide a reliable basis for the interpretation of analogous experiments using human material.

MATERIALS AND METHODS

Animals

Thirty male Wistar Porton rats (Olac Ltd, Bicester) approximately 1 year old and weighing between 450 and 500 g were used in this study. They were maintained under standard conditions; tap water and Breeder's Diet No. 3 (Special Diet Services, Witham, Essex) were provided *ad libitum*. All experiments *in vivo* were begun at 0900 hours to minimise the effect of circadian variation.

In vivo stathmokinetic experiment

Two groups of eleven animals were injected intraperitoneally with vincristine sulphate ('Oncovin', Eli Lilly & Co. Ltd, Basingstoke) at a dose of 1 mg per kg body weight on successive days. Pairs of animals were killed by cervical dislocation at 15 minute intervals thereafter for a duration of $2\frac{3}{4}$ hours. The entire gut from the pyloric region of the stomach to the anus was removed and divided at the ileocaecal junction. The colon was opened along its length, emptied of faecal contents, and pinned luminal surface uppermost to a cork board. After fixation in Carnoy's fluid for 5 hours, transverse blocks were taken from the mid-colon. The tissue was dehydrated through changes of cellosolve to chloroform and processed in paraffin wax. Serial sections 4 μm thick were stained with haematoxylin and eosin.

In vivo mitotic index

Five animals were killed to obtain an estimate of the mitotic index (I_m) *in vivo*. The tissue was processed as described above.

One hundred perfect axially sectioned crypts were located for each animal, and the left hand crypt column, counting from the most basal cell (Position 1) to the crypt-surface junction, was analysed for each crypt. The number of cells in the column and the position of each arrested metaphase or normal mitosis was recorded.

Preparation of tissue for organ culture

Three rats were used for the *in vitro* study and the following procedures, including the stathmokinetic experiments and estimation of mitotic index, were repeated for each of the animals.

The colon was divided at the colocaecal junction, removed, opened along its length, and cleaned of faecal contents. A segment of colon 100–120 mm in length was cut from a region 40 mm proximal to the anal margin. The tissue was transported to the laboratory in ice-cold culture medium.

The mucosa from the isolated segment was stripped from the underlying muscle coat under stereomicroscopic examination. The mucosa was then spread luminal surface uppermost and divided into explants of approximately 2 mm^2. The explants were transferred onto cellulose acetate filters of 3·0 μm pore size (Millipore (UK) Ltd, Harrow) within 60 mm, vented, sterile, plastic petri dishes (Sterilin, Gibco Ltd, Paisley, Scotland). Each dish contained 4 explants.

The standard medium consisted of Waymouth MB 752/1 (Gibco Ltd, Paisley, Scotland) with the addition of 10 % fetal calf serum (Northumbria Biologicals Ltd, Cramlington, Northumberland), 300 μg/ml ascorbic acid (Sigma London, Poole, Dorset), 3 μg/ml hydrocortisone-21-sodium succinate, 0·45 μg/ml ferrous sulphate, 100 units/ml penicillin/streptomycin solution and 100 units/ml mycostatin suspension.

The addition of serum to the culture medium ensures a reliable long-term survival of intestinal mucosa. As the serum composition may vary we would like to eliminate it from the culture medium and replace it with chemically defined supplements; this is obviously desirable when studying the effect of putative trophic factors. The supplements in our supplemented medium are added at levels designed to enhance preservation of the tissue rather than as a direct test of their properties as trophic factors. However, it is possible that their addition does produce changes in proliferative parameters, and the supplemented medium was, therefore, included in this study to check this possibility. This supplemented medium contained in addition sodium butyrate 1 mM (BDH), 1·5 % v/v DMSO, 20 ng/ml epidermal growth factor (EGF), 50 ng/ml glucagon, 5 μg/ml transferrin and 5 μg/ml insulin (all Sigma London, except where stated).

The pH of the medium was adjusted to 7·4 when necessary and was filter-sterilised through a 0·22 μm filter unit (Millipore (UK) Ltd, Harrow). The cultures were rocked at 8 cycles per minute on a rocking platform (Bellco Glass Inc, Vineland, New Jersey) at 37 °C. The medium was changed every 48 hours and the gas phase (95 % O_2/5 % CO_2) renewed every day.

In vitro stathmokinetic experiment

Half of the explants for this study were cultured in the standard medium and the remaining explants received the supplemented medium.

On the seventh day of culture all the explants received 4 μg/ml of vincristine. At 15 minute intervals thereafter, for a duration of $2\frac{3}{4}$ hours, 8 explants from each medium were fixed in neutral buffered formalin. The tissue was processed in the usual manner. Serial sections of 4 μm thickness were cut and stained with haematoxylin and eosin.

In vitro mitotic index

Twenty explants were cultured in standard medium for 7 days and then fixed in formalin.

The natural curvature of the explants and also their small size made it very difficult to obtain optimally orientated sections for counting in the same manner as the *in vivo* tissue. Instead, for each explant, the total number of crypt epithelial cells

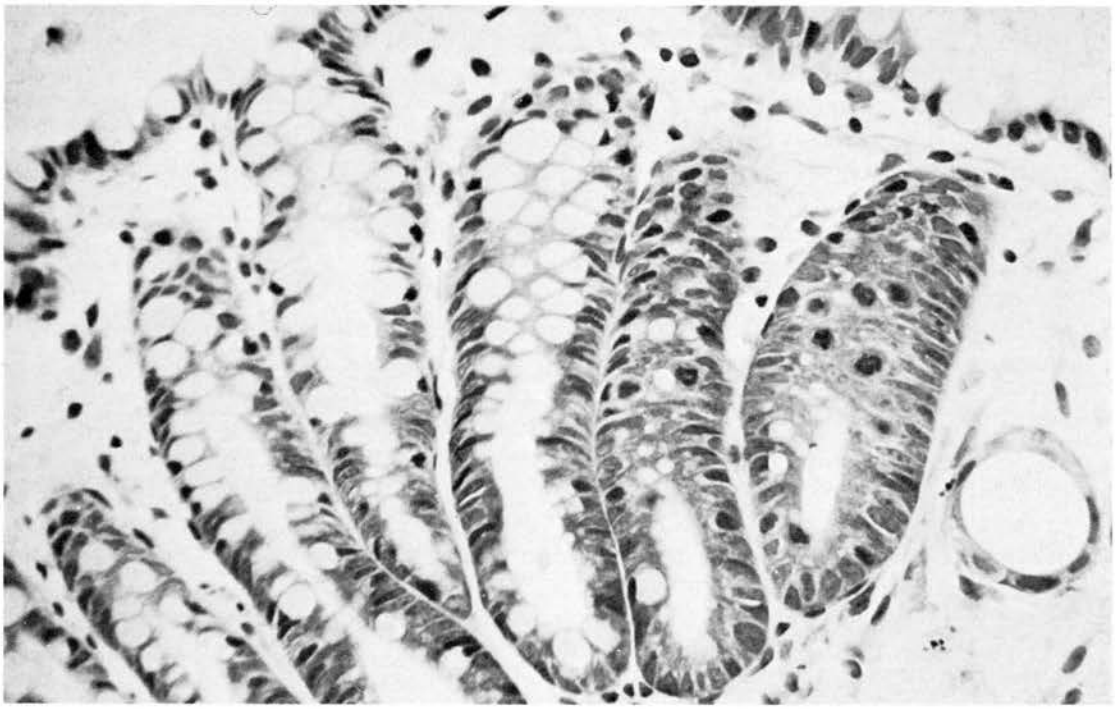

Fig. 1. Edge of explant cultured for 7 days. Crypts in this region appear misshapen and have an increased number of dividing cells. Haematoxylin and eosin. ×400.

in a whole section was counted. Further whole serial sections for each explant were employed if necessary to obtain a minimum of 1000 cells. The number of arrested metaphases in the vincristine experiment and normal mitoses in the mitotic index material was noted.

The analysis of both the *in vivo* and *in vitro* tissues was essentially similar, entailing an estimation of the number of arrested metaphases or normal mitoses occurring within a particular number of crypt cells.

The technique of crypt microdissection was considered for both the *in vivo* and *in vitro* experiments but was not employed for the following reasons. Firstly, microdissection makes it less easy to assess the quality of survival of the explants before making cell counts. Serial sections stained with haematoxylin and eosin ensured that all explants used for cell counts showed good tissue preservation. Secondly, explant variability would necessitate crypt squashes for a larger number of explants at each timepoint thus making the method less efficient than standard histological assessment. Thirdly, the small size of the explants would have rendered the investigation of proliferation at the edge and middle regions extremely difficult (see below). Finally, the analysis of perfect axially sectioned crypts employed *in vivo* provided additional information on the distribution of proliferating cells within the crypt which cannot be gained by crypt squashes.

A study of edge and middle regions within cultured explants

There is a morphological difference between the crypts in the middle and at the edge of cultured explants. The former have a straight, regular test tube-like appearance

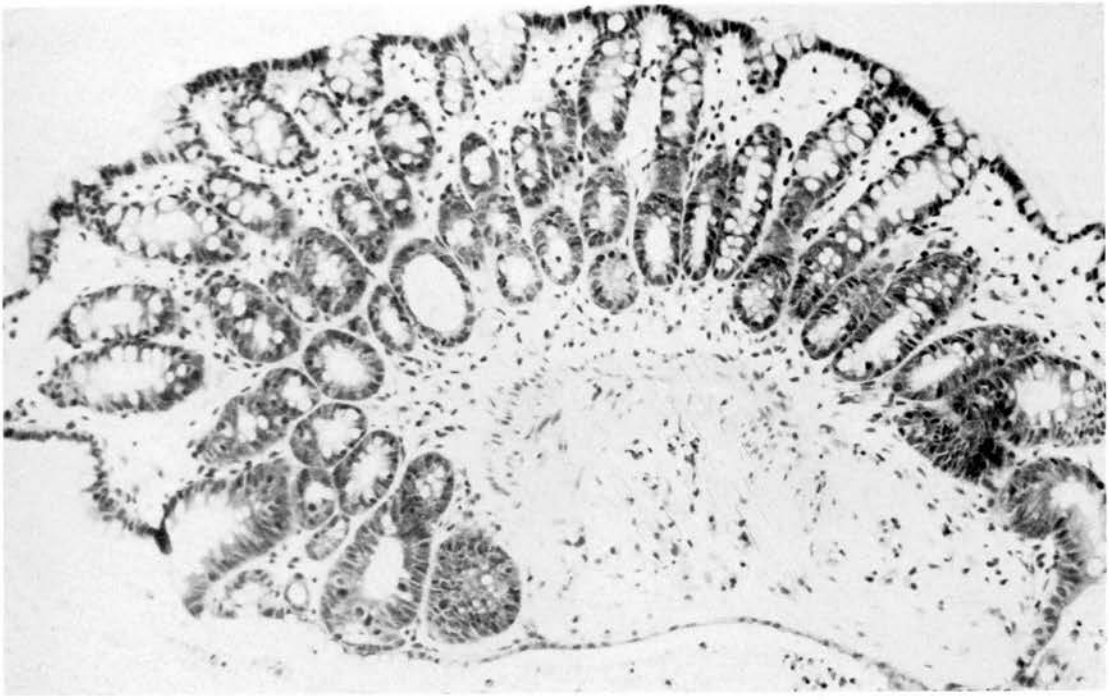

Fig. 2. Explant of colonic mucosa after 7 days in culture showing near-normal morphology. Haematoxylin and eosin. × 150.

with walls of single-cell thickness; the latter consistently appear misshapen, show nuclear crowding and seem to have an increased number of dividing cells (Fig. 1).

To investigate these observations further, explants from two of the experiments were divided into edge and middle regions and counted in the same manner as whole explants. The edge region was defined as the first three crypts encountered, working from the edge of the explant towards the centre. Explants which were too small to enable such a division were not included.

RESULTS

In the *in vitro* experiment, 66 % of the explants showed good tissue preservation, near normal morphology, and no signs of necrosis (Fig. 2), and these were counted. In the majority of explants, however, a few crypts at the edge displayed an increased number of mitotic cells together with a reduction in the number of goblet cells when compared to those in the central region. In addition, many explants possessed a single layer of epithelial cells which, either fully or partially, covered their undersurfaces. No appreciable morphological difference was seen between those explants maintained in the standard medium and those in the supplemented medium.

The individual cell birth rates (k_B) for the 3 animals used in the *in vitro* stathmokinetic experiments are shown in Table 1. Data for the whole explant were pooled to produce values for both the standard and the supplemented medium. However, as no significant difference was found between the birth rates for the two media the data were further combined to produce a single value for the whole explant. Birth rates for the edge and middle regions were obtained by counting two experiments (standard medium only) and again pooling the individual sets of data.

Table 1. *Cell birth rates (k_B) obtained in vitro for standard and supplemented media*

	k_B (cells/1000 cells/hour)			
	Whole explant		Standard medium	
Replicate	Standard medium	Supplemented medium	Edge	Middle
1	5·65	6·97	7·12	3·61
2	5·21	9·88	6·82	2·52
3	11·57	4·45	Not counted	
Pooled data	7·7±0·5 (S.E.)		7·5±0·9 (S.E.)	3·2±0·9 (S.E.)

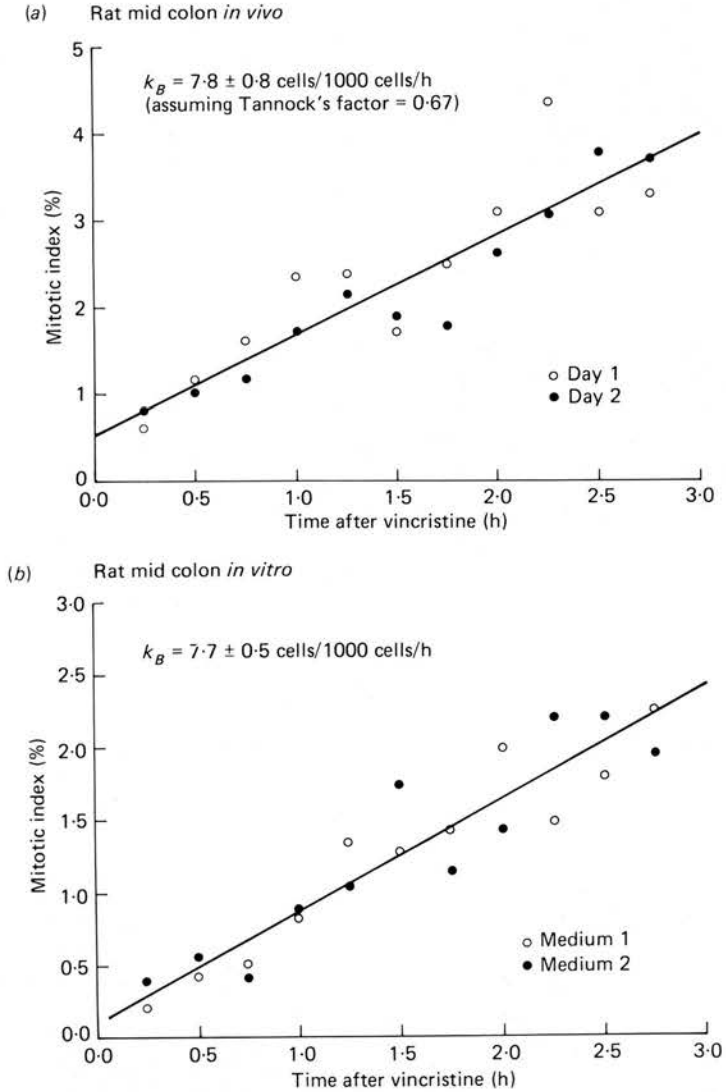

Fig. 3(*a–b*). Cumulative mitotic index plotted against time after vincristine administration for *in vivo* (*a*) and *in vitro* (*b*) stathmokinetic experiments. Birth rate *in vitro* (whole explant) is closely similar to that observed *in vivo*.

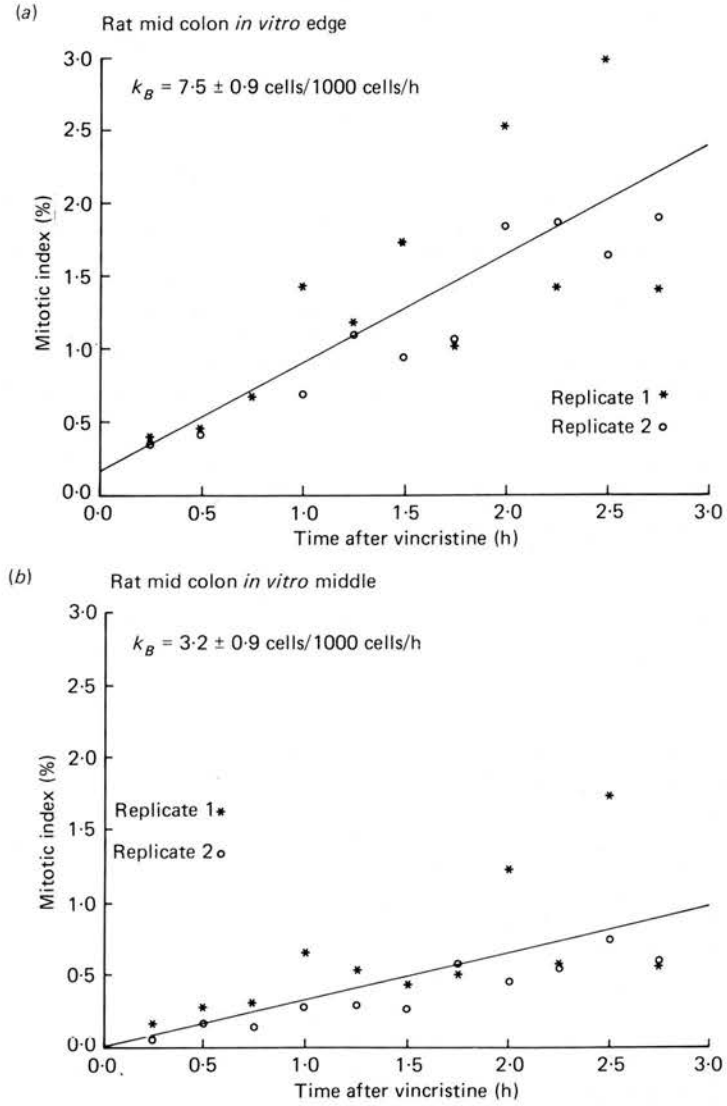

Fig. 4(*a–b*). Cumulative mitotic index plotted against time after vincristine administration for edge (*a*) and middle (*b*) of cultured explants. Birth rates indicate that proliferation differs markedly in these two regions.

Graphical representations of the data from the stathmokinetic experiments both *in vivo* and *in vitro* are shown in Figures 3 and 4 where the accumulation of arrested metaphases has been plotted against time after the addition of vincristine. The lines have been fitted by the method of least squares. If we assume a rectangular age distribution then the slope of the lines produced may be used to estimate cell birth rate.

Table 2 summarises the values for mitotic index (I_m) and birth rate for *in vivo* and *in vitro* tissue. Combining the data for both media in culture gives a k_B of 7·7 ± 0·5 cells/1000 cells/hour for the whole explant which is near the *in vivo* value. In addition, the I_m for the whole explant, although lower, is comparable with the *in vivo* value. *In vivo* values throughout have been corrected by Tannock's factor (Tannock,

Table 2. *Mitotic index (I_m) and cell birth rate (k_B) in vivo and in vitro in rat mid colon*

		In vitro			
	In vivo	Whole explant	Edge	Middle	Middle*
I_m (%)	0·50†	0·38	0·37	0·21	0·13†
k_B (cells/1000 cells/hour)	7·8±0·8	7·7±0·5	7·5±0·9	3·2±0·9	—

* Perfect axial crypt sections. † Corrected by Tannock's factor.

1967) obtained from measurements of cross sections of crypts to compensate for bias in counting axial sections created by the displacement of mitotic figures towards the lumen of the crypt.

The I_m and k_B for the central crypts, however, appear to be approximately half of the value of those found at the edge of the explant. It would be reasonable to expect the k_B for the whole explant to fall between the values for the edge and middle areas. Its failure to do so can be explained by the imprecision of the estimates: the 95 % confidence interval for the k_B at the edge is from 5·7 to 9·3 cells/1000 cells/hour while that for the whole explant is from 6·7 to 8·7 cells/1000 cells/hour.

In one *in vitro* experiment designed to estimate the native mitotic index, crypts were orientated so as to enable 253 perfect axially sectioned crypts in the middle of the explant to be counted; the resulting I_m is given in Table 2. The similarity of this value compared to the I_m obtained from mid sections of explants which were not optimally orientated suggests that data obtained from both methods of analysis do not differ much.

The mean crypt column count obtained for the axially sectioned crypts *in vitro* was found to be 32·9 ± 0·7 (S.E.) cells whilst that *in vivo* was 31·2 ± 1·0 cells, indicating that after 7 days in culture these crypts had not undergone a reduction in length. In addition, the distribution of mitoses within the *in vitro* crypts appeared normal.

DISCUSSION

Our whole explant culture system initially seemed promising as the proliferative parameters appeared to reflect those obtained *in vivo*. However, studies of proliferation in edge and central regions showed that kinetic parameters at the edges were approximately twice those found at the centre.

Several groups of workers have reported increased proliferation at the edges of cultured tissue together with relatively little in the centre (Eastwood & Trier, 1973; Johnson & Bond, 1961; Lieb & Lisco, 1966; Rajewsky, 1965; Shorter, Spencer & Hallenbeck, 1966). In many of these studies human or rodent tumour tissue has been cultured for as little as one hour in a medium containing tritiated thymidine (Hendrickson & Skypeck, 1963; Johnson & Bond, 1961; Lieb & Lisco, 1966; Shorter *et al.* 1966). Steel & Bensted (1965) found that when tumour biopsies were incubated for 30 minutes labelled cells were not seen deeper than 150 μm from the surface of the tissue. Likewise, Johnson & Bond (1961), in a study using mouse tumour, reported that labelling was limited to a peripheral zone of only 8–10 cells (100 μm) thick. In most instances the more intense labelling at the edges has been attributed to an oxygen diffusion defect (Fabrikant *et al.* 1969; Johnson

& Bond, 1961; Meyer & Connor, 1977; Rajewsky, 1965; Steel & Bensted, 1965) resulting in a steep gradient which decreases from the exterior to the interior. The depth of incorporation of tritiated thymidine was reported to be improved by increasing the partial pressure of oxygen in the tissue by the use of hyperbaric oxygen (Denekamp & Kallman, 1973; Fabrikant *et al.* 1969; Meyer & Connor, 1977; Steel & Bensted, 1965) or by bubbling a mixture of 95% oxygen and 5% carbon dioxide through the medium (Johnson & Bond, 1961; Lieb & Lisco, 1966). It was noted that even in tissues *in vivo*, heavier labelling was seen around the areas nearest to blood vessels (Hendrickson & Skypeck, 1963). Despite these problems some groups (Denekamp & Kallman, 1973; Fabrikant *et al.* 1969; Hendrickson & Skypeck, 1963; Meyer & Connor, 1977; Rajewsky, 1965) reported satisfactory uptake of tritiated thymidine, and experiments devised to test the reliability of kinetic data obtained in short-term *in vitro* studies produced good correlation between *in vivo* and *in vitro* values for both tumour and normal mucosa. Experiments in which the tumour was cultured as thin sections provided the most favourable results (Hendrickson & Skypeck, 1963; Meyer & Connor, 1977). In such studies, labelling indices obtained by the incubation of tissue with tritiated thymidine *in vitro* were compared to those obtained by labelling a matched tumour *in vivo*. Although a large regional variation was seen in the percentage of label, both within the tissue and also between the specimens, only those areas of increased labelling were selected for a determination of the labelling index.

Pritchett (1982) and Pritchett *et al.* (1981), in a detailed study of the labelling and mitotic indices in human colorectal tumours and mucosa over a period of 24 hours, reported an early depression of the mitotic index coupled with a heavy uptake of thymidine within 0·3 mm of the cut edge of the samples. However, at 18–24 hours both the labelling and mitotic indices became more uniform and it was suggested that the edge labelling was a local response by the tissue to trauma. These and similar observations show that changes in proliferation occur early in culture, and emphasise the importance of selecting a period of kinetic stability for the assessment of proliferative parameters *in vitro*. Clearly, any results based on counting areas of high labelling when cell proliferation is unsettled will be of doubtful value.

We chose to perform experiments at a time when we expected kinetic indices within the explant to have stabilised. However, the central region of the explants produced values for both mitotic index and birth rate which were approximately half those seen *in vivo* whilst those for the edge were more nearly equal to the *in vivo* findings. It is likely that this difference in proliferation is due to a factor which is more sustained than trauma. It may be that in culture there is a loss of trophic influences necessary for normal *in vivo* levels of proliferation. Perhaps there is an intrinsic basal steady state of cell proliferation *in vivo* at a tissue level in the gut which is periodically modulated by extrinsic factors such as diet (Al-Dewachi, Wright, Appleton & Watson, 1975; Hagemann & Stragand, 1977), bile acids (Deschner & Raicht, 1979), or hormones (Johnson, 1976, 1977). Thus the cultured tissue would display a reduction in proliferative indices to basal levels. At the same time the edges may be stimulated to *in vivo* levels of proliferation by what appears to be an attempt by the crypts at the edge to sustain epithelialisation of the undersurface of the explant.

The phenomenon of epithelial cell outgrowth has been reported previously by other groups. Senior *et al.* (1982) found that the type of support phase used affected

the behaviour of explants in culture. In particular, polycarbonate membranes induced a single layer of epithelial cells to cover the undersurface of the explants. This growth, however, was found not to affect the survival time of the cultured tissue and was attributed to the degree of intimacy of contact between the explant and the support. Likewise, Eastwood & Trier (1973) described the growth of epithelial cells from the peripheral edges of some biopsies. Autoradiography showed labelled columnar cells at the biopsy edges and it was presumed that these were derived from differentiated surface cells.

A similar type of epithelial growth has been described *in vivo* in the human colon (Melnyk, Braucher & Kirsner, 1967) and in that of the dog (Braucher & Kirsner, 1962; Melnyk, Braucher & Kirsner, 1966*a*) and in the ileum (McMinn, 1954) and rectum (McMinn & Johnson, 1958) of the cat. In all cases epithelial cells were seen to migrate from normal glands at the wound margin and to cover the surface of the wound. Histological studies showed a reduction in periodic acid–Schiff (PAS)-positive material in immediate gland epithelium adjacent to the lesion for the first few days of repair (Melnyk, Braucher & Kirsner, 1966*b*). In our system it is possible that the loss of neighbouring crypts in the region of the cut edge may free peripheral tissue from local controlling substances (Bullough, 1965) and stimulate repair mechanisms. The changed morphology of the crypts at the edge, particularly the reduced numbers of mucin-producing cells, suggests a 'dedifferentiation' to facilitate increased cell proliferation.

Despite these observations the culture system may still be regarded as a useful model for study, provided that the topological differences in proliferation which exist within the explants are taken into account. This is particularly important when considering which region of tissue to employ when counting cells and also in the interpretation of results obtained from such a system. The increased proliferation occurring at the edges of the explants suggests that the crypts in these regions are already stimulated and therefore may only have a limited usefulness when studying responses to additional stimuli. On the other hand, the lower level of proliferation found in the centre may provide a basal value from which to monitor responses of the tissue in studies of carcinogenesis or trophic factors.

SUMMARY

The successful development of a long-term organ culture system has made it possible to perform experiments on rat colonic mucosa *in vitro*. However, the effect of trauma or the withdrawal of trophic influences in culture may result in the disturbance of proliferation within the tissue. In this paper we describe an investigation designed to characterise the culture system by a comparison of the proliferative parameters *in vitro* with those *in vivo*.

Stathmokinetic experiments were performed *in vivo* and *in vitro* to estimate cell birth rate. Mitotic indices were also calculated. The *in vivo* birth rate ($7{\cdot}8 \pm 0{\cdot}8$ cells/1000 cells/hour) and the *in vitro* birth rate for the whole explant ($7{\cdot}7 \pm 0{\cdot}5$ cells/1000 cells/hour) were found to be similar. A study of crypts in the centre and at the edge of the cultured explants, however, indicated that proliferation at the two sites differed markedly, the birth rate at the edge ($7{\cdot}5 \pm 0{\cdot}9$ cells/1000 cells/hour) being approximately twice that at the centre ($3{\cdot}2 \pm 0{\cdot}9$ cells/1000 cells/hour).

Provided that this topological difference in proliferation within the explant is recognised the *in vitro* model, in particular the basal level found at the centre, may

still be regarded as a valid system for studying tissue responses to carcinogens and trophic factors.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Kathryn Elliott for preparing the histological sections, Mr S. Brabazon for photographic assistance and Miss E. Wark for typing the manuscript.

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Virchows Arch. B Cell Path. 29, 351–361 (1979)

Virchows Archiv B
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Cell Population Kinetics in the Mucosal Crypts of the Descending Colon of the Mouse

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Summary. In the present study we have used a variety of techniques in order to investigate cell proliferation in the mucosal crypts at a strictly defined site in the descending colon of the mouse.

The mean crypt length was 31.1 cells, and the mean crypt circumference 22.5 cells, giving a total crypt population of about 700 cells. The corrected mitotic index for the whole crypt column was 1.33%; the labelling index was 11.7% with peak values of about 27% occurring at the bottom of the crypt. Metaphase accumulation following vincristine showed that the birth rate in the whole crypt was 26.5 cells/1000 cells/h. From a consideration of cumulative mitotic index and cumulative birth rate a mitotic duration of 0.39 h was obtained.

Analysis of FLM data gave a cell cycle time for the whole crypt of 15.5 h: from FLM data and observed labelling index a growth fraction for the whole crypt of 29% was obtained. Analysis of FLM data for individual cell position groups at different sites within the crypt indicated that the mean cell cycle time showed little variability and was about 15 to 16 h, whereas the duration of DNA synthesis fell with rising cell positions. Growth fraction too appeared to fall with rising cell position within the crypt.

Key words: Colon – Mouse – Kinetics.

Introduction

Cell proliferation in the colonic mucosa of normal mice has been investigated by several groups of workers using a variety of cytokinetic techniques. From examination of material taken from both ascending and sigmoid colon of C57 Brown mice, Lipkin and Quastler (1962) constructed a histogram showing the distribution of labelling activity along the length of the colonic crypt 1 h after

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0340-6075/79/0029/0351/$02.20

the administration of tritiated thymidine (^{3}HTdR). Labelling index (I_s) was highest in the midportion of the crypt, with lower values in the basally situated cells, and tailed off to zero through the upper part of the crypt. The mean I_s for the whole crypt column was 19%, and the distribution of labelled cells was similar to that described by Messier and Leblond (1960) in the ascending colon. In a segment of the descending colon, strictly defined in anatomical terms, Chang and Leblond (1971) showed that the cells at the bottom of the crpyt had high labelling indices when compared with the cells in the midportion of the crypt. This type of distribution is similar to that described by Richards (1977) in the distal colon of normal mice.

The fraction of labelled mitoses (FLM) technique was used by Lipkin and Quastler (1962) in order to estimate the major cell cycle parameters. The mean generation time was of the order of 16 h, and the duration of DNA synthesis (t_s) 6.5 h. The technique was also used by Thrasher (1967) and by Richards (1977), and their estimates of cell cycle time (T_c) for the whole crypt were 19 h and 25–26 h respectively. Chang and Nadler (1975) also using the FLM technique estimated T_c at 23.2 h in three separate types of crypt cell. A less direct estimate was provided by Kovacs and Potten (1973) who performed flash labelling studies on squashed whole crypts and also derived a T_c of 23.2 h, using a value for t_s obtained from the literature and assuming a rectangular age distribution. More recently, these workers have produced a T_c estimate of 18.2 h using a continuous labelling method with ^{3}HTdR (Potten, Kovacs and Hamilton, 1974).

Few estimates have been made of the size of the proliferative compartment within the mouse colonic crypt. Hagemann and Stragand (1977) calculated the size of the proliferative compartment as 37% of the crypt; Kovacs and Potten (1973) estimated that the fraction of cells within the proliferative zone was between 0.35 and 0.52.

The present study has been undertaken in order to examine in detail some of the cytokinetic characteristics of the colonic mucosa. We have used simple morphometry, together with flash labelling and FLM studies with ^{3}HTdR, and also the technique of metaphase arrest with vincristine. Observations in the rat (Sunter, Wright and Appleton, 1978) have shown that there are considerable morphological and cytokinetic differences from one site to another along the length of the colon. Anticipating a similar situation in the mouse we have focussed our attention on a single strictly defined site in the descending colon.

Materials and Methods

Male A2G mice were used throughout. The animals were twelve weeks old when the experiments were performed and weighed 20–25 g. They were fed on 14% rat cake (dried milk) supplied by N.E. Farmers, Aberdeen, and allowed tap water ad libitum. All experiments were commenced at 09.00 h in order to try to avoid distortions caused by the effects of diurnal variation on proliferative indices (Chang, 1971).

Labelling Index Study

Four animals were given tritiated thymidine (Radiochemical Centre, Amersham, England), of specific activity 5 Ci/mMole, by intraperitoneal injection at a dosage of 1 μCi/g body weight. One hour

after the injection the animals were killed by cervical dislocation and the whole colon was removed, opened along its length and pinned to a cork board, mucosal surface uppermost. After fixation for 6 h in Carnoy's solution a transverse block of colon was taken from a site 30% of the total distance from ano-rectal junction to ileocaecal valve. The material was processed through to paraffin wax and autoradiographs were prepared in the usual way from 3 μm thick serial sections, the exposure period being two weeks.

For each animal the "left sides" of one hundred perfect axial sections of crypts were analysed. The total length of the crypt in cells (the crypt column) was recorded, together with the cell positions of mitotic figures and of labelled nuclei. The criterion for identification of labelled cells was the presence of five or more autoradiographic grains located over the nucleus. The data from these four animals were combined, and projected onto a standard crypt the height of which was the mean crypt height of all the columns counted. The thod of Cairnie and Bentley (1967), as modified by Wright, Morley and Appleton (1972), was used to produce labelling and mitotic index distribution diagrams.

Vincristine Study

Eleven animals were given vincristine sulphate ("Oncovin", Eli Lilly) by intraperitoneal injection at a dosage of 1 mg/kg body weight. Animals were killed at 15 min intervals thereafter, the first at 15 min after vincristine and the last at 165 min. The colons were removed and fixed as described above; 3 μm serial sections were prepared from the selected site in the descending colon and stained with Harris's haematoxylin. In each animal the "left sides" of one hundred axially sectioned crypts were analysed; the total crypt height in cells was recorded together with the cell position of arrested metaphases. That metaphase arrest was complete was confirmed by the absence of post-metaphase mitotic figures in material taken 30 min and longer after vincristine. As before, the data were expressed relative to a standard crypt. From an analysis of 50 transverse sections of crypts in each of four animals a correction factor was calculated to compensate for the over-estimation of mitotic index due to migration of mitotic figures towards the lumen of the crypt (Tannock, 1967). In addition counts of the number of cells appearing in transverse sections of crypts (the crypt column count) were made.

FLM Study

Thirty-six animals were injected with ^{3}HTdR at a dosage of 1 μCi/g weight, by the intraperitoneal route. Single animals were killed at hourly intervals up to 24 h after injection, and at two hourly intervals thereafter up to 48 h. Autoradiographs were prepared as described above from the selected site in the descending colon. For the purpose of analysis the crypt was divided into cell position groups, each consisting of four cell positions. The lower group comprised cell positions 1–4, the second group positions 5–8, and so on up the crypt. In each section a minimum of twenty mitotic figures was scored for each of these cell position groups, and the proportion of mitotic figures which were labelled was determined. In this way, for each of the separate cell position groups an FLM curve was constructed, and the data were analysed by the method of Gilbert (1972). An FLM curve for the whole crypt was constructed by calculating the weighted mean of the five separate cell position groups in each animal according to the distribution of mitoses within the crypt.

Results

The mean crypt length (calculated from 1,400 crypts in 14 mice) was 31.1 cells, and the mean column count (from 200 crypts in 4 mice) was 22.5. The product of these two values gives an approximate estimate of the total number of cells per crypt – 700 cells. The mean crypt length in the different animals varied considerably, the standard deviation being 5.4 cells.

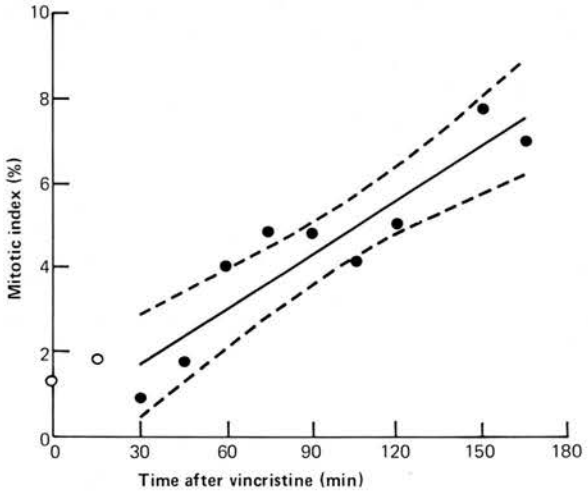

Fig. 1. Mitotic accumulation data for the whole crypt after administration of vincristine. The slope, k_B, of the line fitted to the solid circles is 26.5 ± 4.8 (s.e.) cells/1,000 cells/h. The dotted lines indicate 95% confidence limits for the line

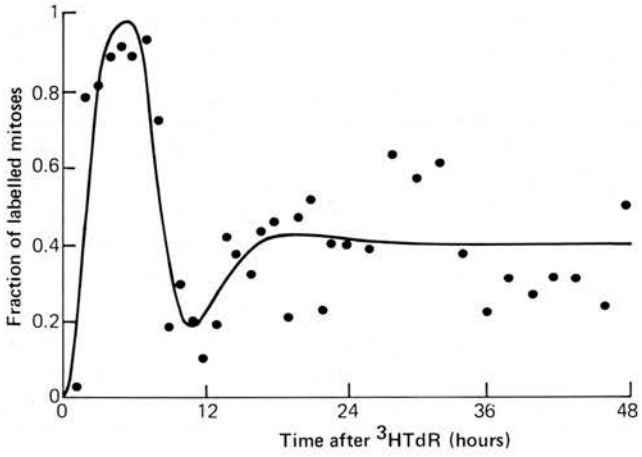

Fig. 2. Fraction of labelled mitoses (FLM) data for the whole crypt. The curve is fitted by the method of Gilbert (1972) and gives $T_c = 15.5$ h with a coefficient of variation of 43%

Figure 1 shows the mitotic accumulation data for the whole crypt following the administration of vincristine; no data were available for the animal killed at 135 min, and readings at less than thirty minutes of treatment were excluded from the analysis as it appears possible that vincristine is not fully effective in arresting cells before this time. We have also observed this phenomenon in the small intestine of the mouse (Al-Dewachi et al., 1975). A straight line was fitted by the method of least squares, and the slope, 26.5 cells per 1,000 cells per hour, was taken to be the birth rate (k_B). In Fig. 1, and wherever the mitotic index is quoted, its value is not that actually observed, but a corrected value which takes into account the artefact mentioned previously; observed indices have been reduced by multiplying by 0.62.

Table 1. Kinetic data for the whole crypt

Variable	Estimated value	How calculated
a) Crypt length (cells)	31.1	
b) Number of columns per crypt	22.5	
c) Total number of cells	700	a × b
d) Corrected mitotic index (%)	1.33	
e) Birth rate (cells/1,000 cells/h)	26.5	from Fig. 1
f) Mitotic duration, t_m (h)	0.50	d/e
g) t_{G1} (h)	7.0	from Fig. 2 and f
h) t_s (h)	6.2	from Fig. 2
i) t_{G2} (h)	1.8	from Fig. 2 and f
j) Cell cycle time, T_c (h)	15.5	from Fig. 2
k) Theoretical labelling index (%)	40	h/j
l) Observed labelling index (%)	11.7	
m) Growth fraction (%)	29	l/k
n) Total number of proliferative cells	200	m × c
o) Cell cycle time, T_c (h)	11.0	m/e

The mean mitotic index (I_m) in the four animals which formed the labelling index group was 1.33%, and the mitotic duration (t_m) can be estimated as 0.50 h from the relationship $t_m = I_m/k_B$. Since the precision of stathmokinetic experiments is poor this estimate will not necessarily be a good one – approximate 95% confidence limits are 0.37 and 0.79 h – and a method of providing a better estimate will be described later.

The data from the FLM experiment for the crypt as a whole are shown in Fig. 2. The curve was fitted by the method of Gilbert (1972), and the durations of the phases of the cell cycle obtained in this way are: $t_{G1} + \frac{1}{2} t_m = 7.2$ h, $t_s = 6.2$ h, $t_{G2} + \frac{1}{2} t_m = 2.1$ h; the cell cycle time (T_c) is 15.5 h. From these estimates of proliferative phase durations the "theoretical labelling index" ($I_s^{theoret}$), which is what the labelling index would be if all the cells were in the proliferative cycle, was calculated. Since the crypt is being considered as a whole it is proper to assume that the age distribution of cycling cells is rectangular and a value of 40% was obtained for $I_s^{theoret}$. The growth fraction (I_p) was calculated from the ratio of the observed labelling index (I_s^{obs}) to $I_s^{theoret}$. The mean value in four animals for I_s^{obs} was 11.7%; hence I_p is 29%.

This value implies that in the average crypt of 700 cells 200 are proliferative. It may also be used in the formula $T_c = I_p/k_B$ to give another estimate of the cell cycle time, but again this method is inherently unreliable: even if I_p were known exactly, the uncertainty in k_B would give 95% confidence limits for T_c of 8 h and 17 h; the mean is 11.0 h. All the results presented so far are summaried in Table 1.

Figure 3 illustrates the pooled labelling and mitotic index data from the four flash labelling animals in a crpyt standardised to the mean crypt length. If t_m were known then we could calculate k_B at each position from the mitotic index data using the equation $k_B = I_m/t_m$. Assuming that t_m does not change with cell position we may therefore calculate the cumulative birth rate as the

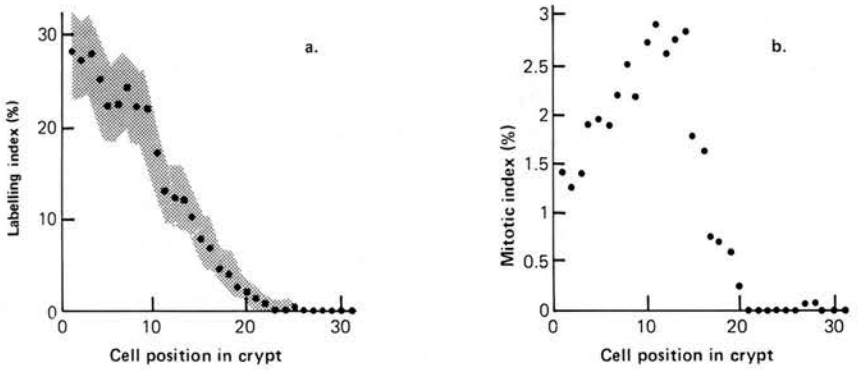

Fig. 3. a Labelling index and **b** mitotic index as a function of cell position in the crypts. The shaded area indicates 95% confidence intervals for the points

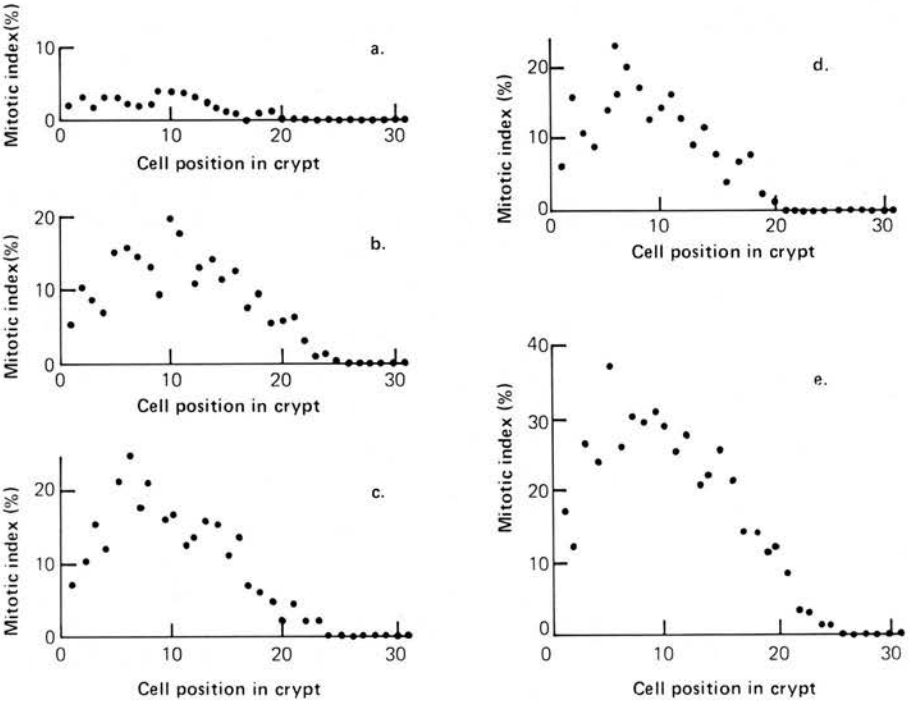

Fig. 4a–e. Mitotic index as a function of cell position in the crypt **a** 30 min, **b** 60 min, **c** 90 min, **d** 120 min, and **e** 150 min, after administration of vincristine

cumulative mitotic index divided by t_m: for our data this is $0.36/t_m$. But we may also calculate the birth rate at each cell position by considering separate stathmokinetic experiments to have been performed at each position. Fig. 4 shows examples of the mitotic index distribution in the crypt at several times after the administration of vincristine; from these distributions, and those at

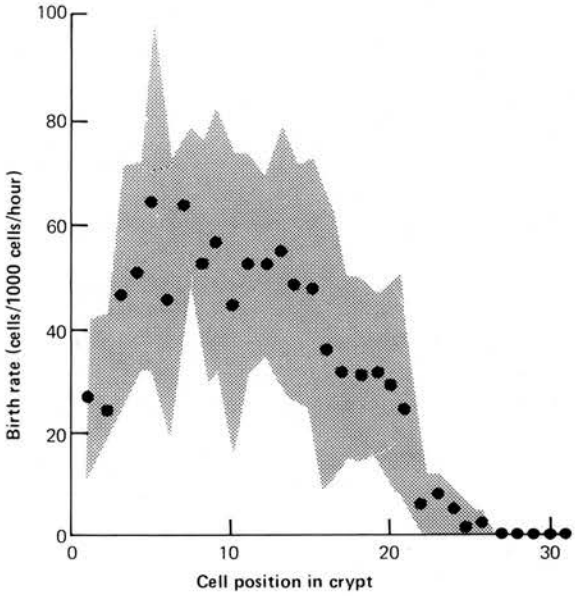

Fig. 5. Birth rate, k_B, as a function of cell position in the crypt. The shaded area indicates 95% confidence intervals for the points

Table 2. Kinetic data from the mitotic index at each cell position

Variable	Estimated value	How calculated
p) Cumulative mitotic index (cells/column)	0.36	from Fig. 3b
q) Cumulative birth rate (cells/column/h)	0.93	from Fig. 5
r) Mitotic duration, t_m (h)	0.39	p/q
s) Crypt cell production rate (cells/h)	21	$q \times b$

Table 3. Kinetic data from the labelling index and FLM in cell position groups

Variable	Cell position group				
	1–4	5–8	9–12	13–16	17–20
t_{G1} (h)	5.7	6.2	7.5	8.2	7.3
t_s (h)	7.6	6.7	5.8	5.7	5.4
t_{G2} (h)	1.5	1.6	2.1	2.0	2.3
t_m (h) (assumed)	0.4	0.4	0.4	0.4	0.4
T_c (h)	15.3	14.9	15.8	16.3	15.4
$I_s^{theoret}$ (%)	45	40	32	30	31
I_s^{obs} (%)	27.0	22.8	16.0	9.2	3.3
I_p (%)	60	57	49	31	11

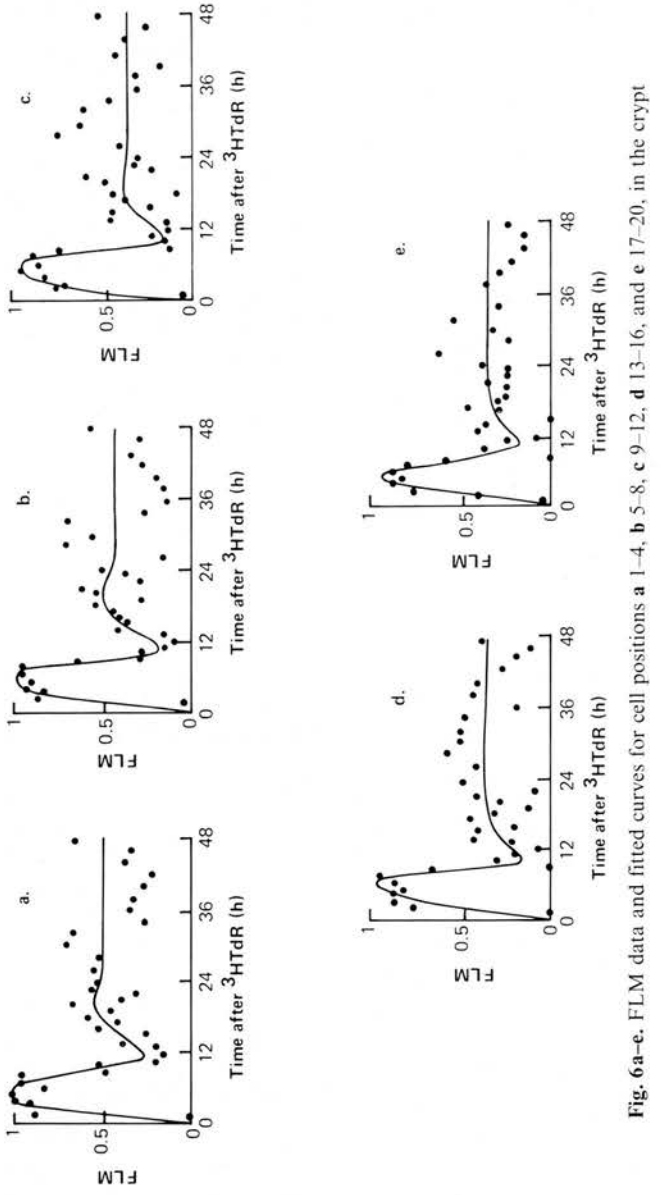

Fig. 6a–e. FLM data and fitted curves for cell positions **a** 1–4, **b** 5–8, **c** 9–12, **d** 13–16, and **e** 17–20, in the crypt

the other times, and assuming an exponential age distribution at each cell position, Fig. 5 was obtained. This shows the birth rate at each cell position, and from it a value of 0.93, with a standard error of 0.05 is obtained for the cumulative birth rate. Combining these two methods gives $0.36/t_m = 0.93$, whence $t_m = 0.39$ h. Confidence limits may be derived from this estimate, though as usual their validity depends on that of the assumptions made regarding age distributions; formal 95% confidence limits are 0.35 and 0.44 h. The

cumulative birth rate may be multiplied by the column count to show that each crypt produces about 21 new cells per hour. Table 2 summarises these results.

The analyses of the FLM experiment by the 4-cell position groups are presented in Table 3; the original data and the fitted curves are shown in Fig. 6. As with the crypt as a whole we have calculated the $I_s^{theoret}$ values (this time assuming exponential age distributions) and the estimates of growth fraction have been obtained by dividing by these the appropriate observed labelling indices derived from the labelling index distribution diagram, Fig. 3a. Because the estimates of t_s decrease with rising cell position so do the values for $I_s^{theoret}$, and hence the estimates of I_p, though decreasing from 60% at positions 1–4 to 11% at positions 17–20, fall relatively more slowly than the observed labelling indices. These calculations of individual growth fractions imply that about 27% of the cells in the crypt as a whole are proliferating, a value which agrees closely with the 29% estimate already obtained from the consideration of whole crypt data.

Discussion

The mean crypt length at this site in the descending colon is 31.1 cells, and the column count is 22.5 cells. The product of these two values gives an estimate of the total crypt cell population. This is 700 cells, which is considerably greater than the count of 358 cells observed by Kovacs and Potten (1973) obtained in whole crypt squashes. These authors commented on the lower labelling indices found in the bottom of the mouse crypt, compared with indices found higher up the crypt. This sort of distribution of label was described by Lipkin and Quastler (1962), who used material from the ascending colon, and found a much shorter crypt than we did; their mean value was 20.3 cells. On the other hand, both Chang and Leblond (1971) and Richards (1977) observed a similar distribution of thymidine label to that described in the present study, and mean crypt heights – 29 cells and 29.3 cells respectively – which were very similar. These authors were using material from the descending colon. It is possible that the differences mentioned may simply represent strain differences; but we feel that it is more likely that there is some major difference in crypt size and kinetic organisation from one site to another in the mouse colon. Such differences do exist in the rat (Sunter et al., 1978). The overall I_s in our material was 11.7%, a figure which is greater than that observed by Richards (1977) but rather lower than that observed by other authors (Lipkin and Quastler 1962; Kovacs and Potten 1973); peak labelling indices are similar. In the small intestine of the mouse Al-Dewachi and co-workers (1975) described peak labelling indices twice as great, of the order of 60%.

The analysis of the FLM study produced an estimate of T_c of 15.5 h for the whole crypt, with a t_s of 6.2 h. These figures agree very closely with the corresponding estimates of Lipkin and Quastler (1962). Thrasher (1967) estimated T_c to be 19 h, while Chang and Nadler (1975) calculated 23.2 h; both these authors used the FLM technique. Our figures are also remarkably close

to estimates of these parameters in the mouse small bowel (Al Dewachi et al., 1975). This contrasts with the situation in the rat where the T_c of colonic crypt epithelium is much prolonged by comparison with that in the jejunum (unpublished observations).

We have analysed FLM data for different cell position groups within the crpyt, and while it appears that the mean cell cycle time remains fairly constant at around 15–16 h, t_s falls slightly with rising cell position within the crypt. We have found no evidence of a substantial subpopulation of basally situated slowly cycling cells – this is in contrast to the small intestine of the mouse (Al-Dewachi et al., 1975) and the small and large intestine of the rat (Wright et al., 1972; Sunter et al., 1978).

The growth fraction of the whole crypt was estimated as 29% from labelling index data and the parameters of the cell cycle derived from the analysis of the FLM curve. Kovacs and Potten (1973) determined the proliferative fraction to be between 0.35 and 0.52, while Hagemann and Stragand (1977) estimated the proliferative compartment size as 37% of the total crypt cell population. Our detailed analysis of labelling indices and fraction of labelled mitoses shows that the growth fraction falls from 60% at the bottom of the crypt to only 11% at positions 17–20.

When analysis of the stathmokinetic experiment provides an estimate for k_B of 26.5 cells/1,000 cells/h. The cumulative birth rate is 0.93 cells/column/hour. Dividing this figure by the cumulative mitotic index gives an estimate of mitotic duration of 0.39 h. The total crypt cell production rate is 21 cells per hour.

Acknowledgements. This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss E. Wark who typed the manuscript, and Mrs. E. Wallace and Mrs. M. Hughes who provided technical assistance. Mrs. G. Bainbridge kindly prepared the illustrations.

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Received October 3, 1978

David Appleton

J. Anat. (1979), **129**, 4, pp. 833–842
With 5 figures
Printed in Great Britain

A comparison of cell proliferation at different sites within the large bowel of the mouse

22

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(*Accepted* 2 *January* 1979)

INTRODUCTION

The morphometric and cytokinetic characteristics of the colonic mucosa of normal mice have been described in a number of independent reports. A comparison shows that there is often marked inconsistency between the observations of different groups of workers. For example, Chang & Leblond (1971) calculated a mean crypt height of 29 cells, whereas the estimate of Lipkin & Quastler (1962) was only 20 cells. The latter authors described the distribution within the crypt of tritiated thymidine (^{3}HTdR) labelling activity, and found the highest labelling indices in the middle portion of the crypt, with lower values at the crypt bottom, a distribution similar to that described by Messier & Leblond (1960). On the other hand, Chang & Leblond (1971) and Richards (1977) have described labelling indices which were highest at the bottom of the crypt, and fell steadily with rising cell position. Similarly estimates of cell cycle time (T_c) have ranged from about 16 hours (Lipkin & Quastler, 1962) to 27 hours (Galand, Rodesch, Leroy & Chrétien, 1967).

In the rat we have previously described considerable differences in the cytokinetic organisation of the crypts from one site to another along the length of the colon (Sunter, Wright & Appleton, 1978), differences which accompany changes in the morphology of the mucosa.

There is a similar pattern of morphological change in the mouse colon, and these differences may in themselves be sufficient to account for some of the apparent inconsistencies in the literature. This study was undertaken to compare the morphological and proliferative characteristics of the mucosa at different levels within the colon. We have used cytokinetic studies with ^{3}HTdR, and the metaphase-arresting agent vincristine, as well as simple morphometric techniques.

MATERIALS AND METHODS

Male A2G mice weighing 20 to 25 g and aged 12 weeks were used throughout. They were fed on 14 % rat cake (dried milk) supplied by N.E. Farmers, Aberdeen, and were allowed access to tap water *ad libitum*. In order to minimise the effects of diurnal variation on proliferative indices (Sigdestad, Bauman & Lesher, 1969; Chang, 1971) all experiments were commenced at 0900 hours.

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Native mitotic and labelling indices

Four animals were given tritiated thymidine (Radiochemical Centre, Amersham, England) of specific activity 5 Ci/mMole, by intraperitoneal injection at a dosage of 1 μCi/g body weight. One hour after the injection the animals were killed by cervical dislocation, and their colons including the caeco-appendix removed in continuity. The colon was opened along its length, cleaned and pinned, mucosal surface uppermost, to a cork board. After fixation for 6 hours in Carnoy's solution, four transected segments of bowel were taken from four representative sites defined and denoted as follows:

(i) 30 % of the distance from anus to ileocaecal valve ('descending colon');
(ii) 60 % of the distance from the anus ('transverse colon');
(iii) 90 % of the distance from the anus ('ascending colon');
(iv) At the junction of distal and middle thirds of caeco-appendix ('caecum').

These blocks were processed through to paraffin wax in the usual way, and autoradiographs were prepared on 3 μm thick serial sections using a dipping technique, the exposure period being 2 weeks.

From each sample the 'left sides' of 100 axially sectioned crypts were analysed. The total length of each crypt in cells (the crypt column) was recorded together with the cell positions of mitotic figures (late prophases, metaphases and early anaphases) and of labelled cells (those with five or more autoradiographic grains over the nucleus). For each of the selected sites within the bowel, diagrams of mitotic and labelling index distribution were produced by combining the data from the 4 animals and projecting them onto a standard crypt, the length of which was the mean of all the crypt columns counted in each site. A modification (Wright, Morely & Appleton, 1972) of the computer method of Cairnie & Bentley (1967) was used.

Vincristine study

Eleven animals were given an intraperitoneal injection of vincristine sulphate ('Oncovin', Eli Lilly) at a dosage of 1 mg/kg body weight. Animals were killed at 15 minute intervals thereafter, the first at 15 minutes after the injection and the last at 165 minutes. The colons were fixed in Carnoy's solution and samples taken from each of the four separate sites described previously. Serial 3 μm sections were prepared and stained with Harris' haematoxylin. In each sample the 'left sides' of 100 axially sectioned crypts were analysed. Total crypt length in cells was recorded together with the cell positions of arrested metaphase figures. Adequacy of metaphase arrest was confirmed by the absence of any post-metaphase mitotic figures in material taken 30 minutes and longer after the administration of vincristine. As before, the data were projected onto standard crypts. In 4 animals from this group counts of the number of cells appearing in transverse sections of crypts were made (the crypt column count); 50 transversely sectioned crypts were counted for each site in the colon and in each of the 4 animals. Analysis of these transverse sections also allowed calculation of a correction factor to compensate for over-estimation of mitotic index caused by displacement of mitotic figures towards the lumen of the crypt (Tannock, 1967).

Fraction of labelled mitoses (FLM) study

Thirty six animals were given ^{3}HTdR at a dosage of 1 μCi/g body weight by intraperitoneal injection. Thereafter animals were killed at hourly intervals up to 24 hours, and then at 2 hourly intervals up to 48 hours. Autoradiographs were prepared as described previously for each of the selected sites within the bowel. For the purpose of analysis the crypts were divided into cell position groups each consisting of 4 cell positions. At the bottom of the crypt cell positions 1 to 4 made up the first group; the second group comprised positions 5 to 8 and so on up the crypt. In each section a minimum of 20 mitotic figures was counted for each of the cell position groups and the proportion which was labelled was determined. Thus for each of the cell position groups an FLM curve was constructed and the curves were analysed by the method of Gilbert (1972). From the data comprising these individual curves an FLM curve for the 'whole crypt' in each of the sites within the colon was constructed by calculating mean values weighted according to the distribution of mitotic figures within the crypt.

RESULTS

The mean crypt length and the mean crypt column count for each of the four sites along the length of the bowel are shown in Table 1. In the descending colon (i) and transverse colon (ii) crypts are of a similar length, 31 cells and 35 cells respectively, whereas in the ascending colon (iii) the crypts are much shorter, 19 cells; in the caecum the crypts are of an intermediate length, 25 cells, but are of a greater circumference, as indicated by the high crypt column count. An approximate estimate of the total number of cells per crypt has been arrived at by calculating the product of these two measurements. In the ascending colon (iii) the figure for total crypt population is about half that of the other sites.

Figure 1 shows the mean mitotic index, I_M for untreated animals as the reading at time zero in each of the four diagrams corresponding to the four sites in the bowel. This value, like all other mitotic indices we shall give, has been corrected to take account of the over-estimation of I_M found by counting axial sections of crypts (Tannock, 1967). The factors by which observed indices have been multiplied are 0·62, 0·53, 0·57 and 0·61 for the sites (i), (ii), (iii) and (iv) respectively. The data from the stathmokinetic experiment treating the crypt as a whole are also presented in Figure 1; the solid lines have been fitted by least squares to the points from 30 minutes onwards when we are sure that vincristine is fully effective. If the age distribution for the total proliferative population of the crypt is rectangular the slopes of these lines are estimates of the cell birth rate, k_B; the mitotic duration, t_M can be found, albeit imprecisely, from the ratio of I_M to k_B and is given in Table 1 along with the values of these other variables.

The results of the FLM experiment for the crypts as a whole, and the curves fitted (Gilbert, 1972), are shown in Figure 2. The estimates of the phase durations are detailed in Table 1; those for G_1 and G_2 have each been reduced from the Gilbert estimates by $\frac{1}{2}t_m$, since the program assumes mitosis to be an instantaneous event and hence must over-estimate t_{G1} and t_{G2}. The quality of the fit is not good, and this is explicable at least partly on the basis of dilution of the thymidine label through several cell divisions, since the first peak and the first half of the second peak are well defined in all the bowel sites. Cell cycle time estimates vary from 15·5 hours at site (i), the descending colon, to 21·2 hours at site (ii), the transverse colon. From these

Table 1. *Kinetic data for the whole crypt*

Variable	Site in the bowel (i)	(ii)	(iii)	(iv)
Crypt length (cells)	31·1	34·7	19·3	25·3
Number of columns per crypt	22·5	19·6	16·2	25·0
Total number of cells	700	680	310	630
Observed mitotic index (%)	1·33	0·84	1·16	1·09
Birth rate (cells/1000 cells/hour)	26·5	12·9	16·2	17·6
Mitotic duration t_m (hour)	0·50	0·65	0·72	0·62
tG_1 (hour)	7·0	10·3	9·4	6·7
t_s (hour)	6·2	8·8	7·4	6·8
tG_2 (hour)	1·8	1·4	1·4	1·2
Cell cycle time T_c (hour) (FLM study)	15·5	21·2	18·9	15·3
Theoretical labelling index (%)	40	42	39	44
Observed labelling index (%)	11·7	12·1	12·3	13·5
Growth fraction (%)	29	29	31	30
Total number of proliferative cells	200	200	100	190
Cell cycle time T_c (hour) (vincristine study)	11·0	22·5	19·4	17·2

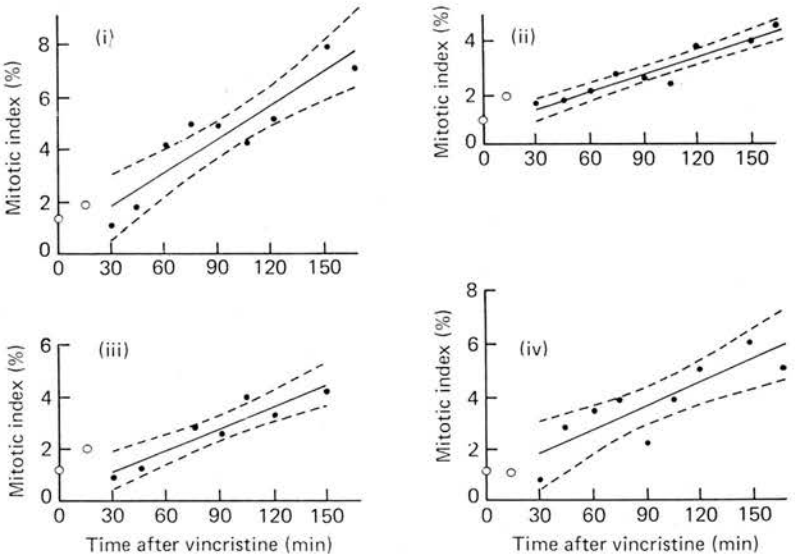

Fig. 1. Mitotic accumulation data for the whole crypt after administration of vincristine. The birth rates were estimated from the slopes of the lines fitted to the solid circles. The dotted lines indicate 95 % confidence limits for the lines. Figs. (i), (ii), (iii) and (iv) correspond to bowel sites (i), (ii), (iii) and (iv) as described in the text.

cell cycle parameters a 'theoretical labelling index' I_s^{theoret}, may be calculated, and from the ratio of the actual labelling index, I_s^{obs}, to I_s^{theoret}, we may estimate the growth fraction, I_p. From the growth fraction and the total number of cells in the crypt the number of proliferative cells in the crypt can be calculated; and from the growth fraction and the birth rate (derived from the stathmokinetic study) a further estimate of T_c is obtained. It should be noted that both these estimates of T_c involve the FLM data, and that the degree to which they agree is a measure of the consistency of the labelling index and stathmokinetic results. The estimated values for all these variables appear in Table 1.

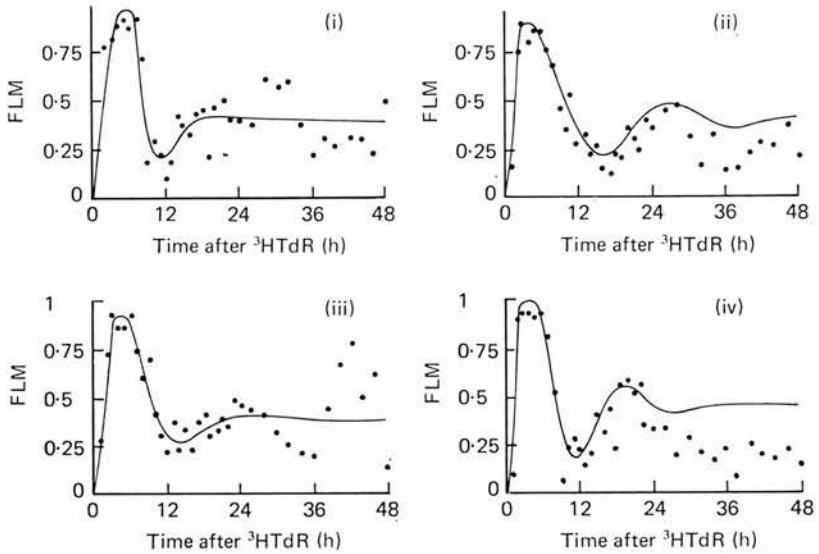

Fig. 2. Fraction of labelled mitoses (FLM) data for the whole crypt fitted by the method of Gilbert (1972). Figs. (i), (ii), (iii) and (iv) correspond to the sites described in the text.

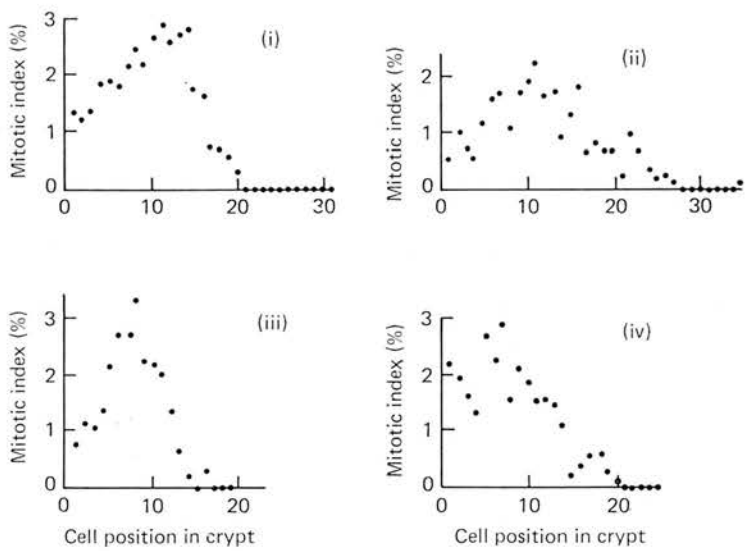

Fig. 3. Mitotic index distribution in the crypt. Figs. (i), (ii), (iii), and (iv) correspond to the sites described in the text.

Figure 3 illustrates the mitotic index at each cell position in the crypt at each of the four sites along the length of the bowel. The results have been related to the mean crypt length relevant to each site. As described in our separate report on the descending colon of the mouse (Rodriguez *et al.* 1979) it is possible to obtain a more reliable estimate of t_m by dividing the cumulative mitotic index by the cumulative birth rate. The data giving rise to the four cumulative birth rates are extensive, comprising 110 separately treated stathmokinetic experiments, and are not presented here, but the cell birth rates estimated by assuming an exponential age distribution

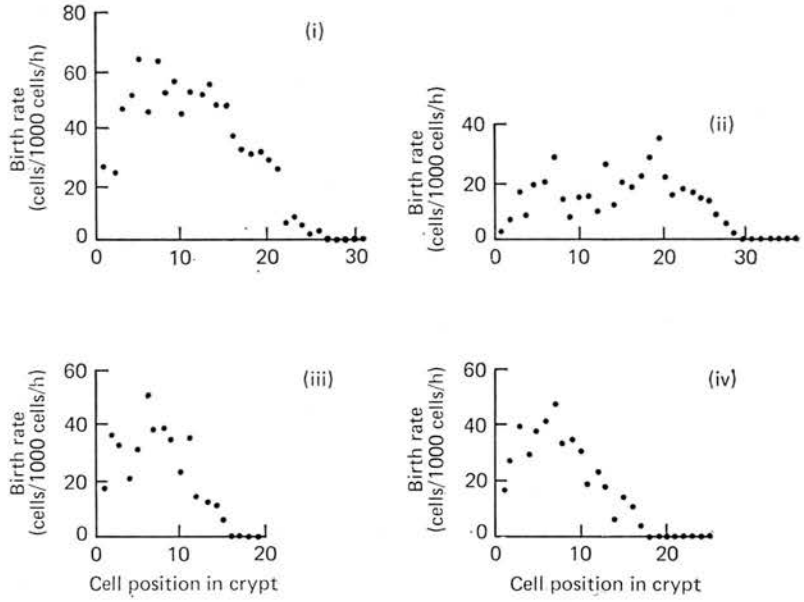

Fig. 4. Birth rate as a function of cell position in the crypt. Figs. (i), (ii), (iii) and (iv) correspond to the sites described in the text.

Table 2. *Kinetic data from mitotic index at each cell position*

Variable	Site in the bowel			
	(i)	(ii)	(iii)	(iv)
Cumulative mitotic index (cells/column)	0·36	0·28	0·25	0·28
Cumulative birth rate (cells/column/hour)	0·93	0·44	0·36	0·43
Mitotic duration t_m (hour)	0·39	0·64	0·69	0·65
Crypt production rate (cells/hour)	20·9	8·6	5·8	10·8

at each position in the four standard crypts are shown in Figure 4. The values of the cumulative birth rates resulting from summation of these individual rates are given in Table 2, along with the cumulative mitotic indices and the estimates of mitotic duration. The cell production rates per crypt, from the product of column counts and cumulative birth rates, are also given. The greatest crypt cell production rates are seen in the descending colon, while the lowest values are seen in the shorter crypts in the ascending colon.

Figure 5 shows the labelling index distribution diagrams for each of the 4 sites along the length of the bowel; they have been calculated from the same group of animals as that used to give the mitotic index distribution diagrams illustrated in Figure 3. In the descending colon, (i), the highest I_s values, of the order of 25 to 30 %, are found at the bottom of the crypt, with a steady fall in I_s with rising cell position. In the ascending colon, (iii), on the other hand, peak I_s is found at the middle of the crypt, with lower values at the crypt bottom. The transverse colon, (ii), shows an intermediate sort of pattern, while in the caecum the highest values of I_s are considerably greater than elsewhere, and are found near the base of the crypt.

The 18 FLM curves resulting from treating each group of four proliferative crypt cells separately have not been illustrated, but the estimated cell cycle times are

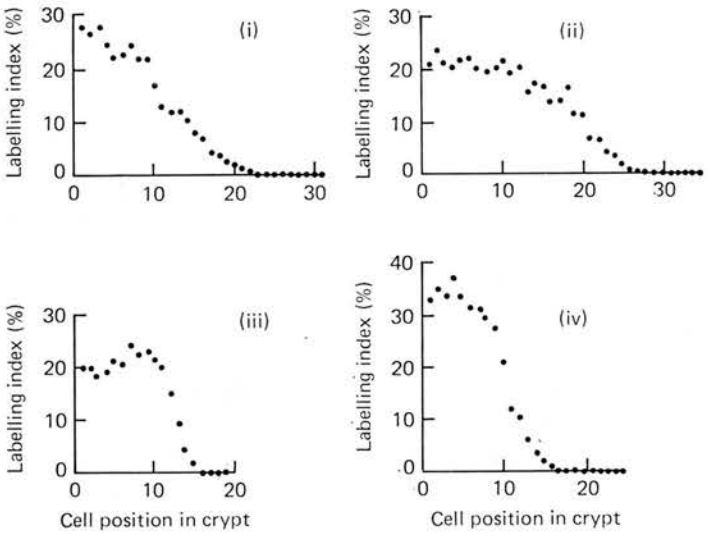

Fig. 5. Labelling index distribution in the crypt. Figs. (i), (ii), (iii) and (iv) correspond to the sites described in the text.

Table 3. *Kinetic data from labelling index and FLM in cell position groups*

		(*a*) Cell cycle time T_c (hour) Cell position group				
		1–4	5–8	9–12	13–16	17–20
Site in bowel	(i)	15·3	14·9	15·8	16·3	15·4
	(ii)	22·0	21·7	22·0	20·9	20·1
	(iii)	20·6	18·2	20·4	20·0	
	(iv)	15·3	15·5	16·0	12·9	
		(*b*) Growth fraction (%)				
Site in bowel	(i)	60	57	49	31	11
	(ii)	55	58	55	48	44
	(iii)	55	62	65	13	
	(iv)	92	84	47	10	

detailed in Table 3*a*. There are no obvious differences in T_c, however, between one cell position group and another within the crypts in any of the sites selected. From the labelling index observed (Fig. 5), and the theoretical labelling indices obtained from the estimates derived from the fitted FLM curves, we can calculate the I_p for each of the cell position groups at each site in the bowel; these are detailed in Table 3*b*. Fuller details of this and other calculations, together with some error estimates, may be found in Rodriguez *et al.* (1979).

DISCUSSION

In a previous report (Rodriguez *et al.* 1979) we have published some observations on the morphometry and cytokinetic organization of the crypts in the descending colon of normal mice. While many of these results showed a good agreement with the published work of some other authors, there were also a number of inconsistencies. We suggested that some of these apparent inconsistencies were

explicable on the basis of differences in the morphology and the proliferative behaviour from one site to another along the length of the mouse colon. Such differences have been observed in the colonic mucosa of the rat (Sunter *et al.* 1978). The present study was undertaken to establish whether or not a similar situation exists in the mouse.

We have calculated in the descending colon a mean crypt length of 31 cells. This agrees closely with the figures of Chang & Leblond (1971) and Richards (1977), whose estimates were 29 cells and 28 to 32 cells respectively. In both of these communications the site in the descending colon from which the material was taken was described in precise anatomical terms. In the transverse colon we have found that the mean crypt length is slightly greater, at 35 cells, whereas in the ascending colon the figure is only 19 cells. This is close to the value of 20 cells obtained by Lipkin & Quastler (1962), who used material from both ascending and sigmoid colon, although they do not state whether they observed any morphological differences between the 2 sites. In the caecum we have found that the mean crypt length is 25 cells. Our approximate estimates of the total crypt population, based on the product of crypt length and crypt column count, show that the crypts in the ascending colon, with a mean population of 310 cells, are about half the size of the crypts at the other sites investigated. This figure is close to that of 358 cells calculated from whole crypt squashes by Kovacs & Potten (1973). These authors do not specifically mention the site from which their material was taken, but they describe and illustrate crypts in which the basally situated cells show lower labelling indices than the cells in the mid-portion of the crypt. This is the pattern of labelling that we have demonstrated in the small crypts of the ascending colon. A similar sort of pattern was described by Messier & Leblond (1960) and by Lipkin & Quastler (1962) in the ascending colon. The distribution of labelled nuclei in the crypts of the descending colon, on the other hand, is quite different, with peak labelling indices at the bottom of the crypt, falling steadily with rising cell positions. This is the sort of pattern described by Chang & Leblond (1971) and Richards (1977) in their studies on the descending colon. In the transverse colon we have observed that there is an intermediate pattern of distribution of labelled cells.

From the analysis of FLM data we have derived estimates of T_c for the whole crypt at each of the sites in the bowel. The mean values are 15·5 hours in descending colon, 21·2 hours in transverse colon, 18·9 hours in ascending colon and 15·3 hours in the caecum. These values are similar to those obtained from analysis of the stathmokinetic experiment, after taking the growth fraction into consideration. A number of T_c estimates based on FLM data are to be found in the literature. Lipkin & Quastler (1962) estimated T_c as 16 hours in ascending colon; Thrasher's (1967) estimate was 19 hours for descending colon, while Chang & Nadler (1975) calculated 23 hours at this site. Using a double labelling technique Galand & co-workers (1967) calculated a T_c of 28 hours in normal mice, and using continuous labelling, Potten, Kovacs & Hamilton (1974) produced an estimate of 18 to 20 hours. We have analysed FLM curves for separate cell position groups within the crypts, and in none of the sites have we found any major difference in T_c between one group of cells and another within the same crypt, though any difference which might exist would tend to be masked by cell migration during the experiment.

Kovacs & Potten (1973) calculated that between 35 and 52 % of colonic crypt cells were in the 'proliferative zone', while Hagemann & Stragand (1977) estimated the 'proliferative compartment size' as 37 % of the total crypt cell population. We

have estimated I_p for the whole crypt from labelling index data and the cell cycle parameters calculated from the FLM experiment. The values are virtually the same – around 30 % – in all 4 sites examined. This figure is lower than those of the authors mentioned because, within the zone of the crypt broadly termed the proliferative compartment, I_p is by no means 100 %. Estimates of I_p by cell position group are detailed in Table 3*b*; differences in the distribution of proliferating cells are reflected in the labelling index distribution diagrams (Fig. 5). In absolute terms the crypts in the ascending colon contain about 100 proliferative cells, whereas in the other sites the figure is around 200 cells. The crypts in the descending colon have the highest cell production rate (21 cells/crypt/hour), while in the ascending colon crypt cell production rate is smallest (6 cells/crypt/hour).

In conclusion, we feel that this study has confirmed our suggestion that important cytokinetic differences exist from one site to another within the mouse colon, the differences are of a similar nature to those we have observed in the rat although less pronounced.

SUMMARY

This study was undertaken in order to compare cell proliferation at different sites along the length of the large bowel of the mouse. Simple morphometry has been used along with ^{3}HTdR labelling studies and metaphase arrest with vincristine. Differences have been described in the shape and size of crypts, the distribution of proliferating cells, the duration of the cell cycle, as well as in the rate of cell production by the crypts. The findings explain some of the apparent inconsistencies in the literature.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss E. Wark and Miss P. Barratt for secretarial assistance, and Mrs E. Wallace and Mrs M. Hughes who prepared the histological sections. Mrs G. Bainbridge kindly prepared the illustrations.

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Cell Proliferation in the Gastrointestinal Tract

23

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PITMAN MEDICAL

1.4 Cell Proliferation in the Mouse Large Bowel, with Details of the Analysis of the Experimental Data

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1.4.1 INTRODUCTION

The morphometric and cytokinetic characteristics of the colonic mucosa o normal mice have been described in a number of reports, but there are apparen discrepancies between the findings of different groups of workers. For example the mean crypt height has been reported as 20 cells (427) and 29 cells (95); the tritiated thymidine (^{3}HTdR) labelling activity has been found to be highest in the middle portion of the crypt (427, 472), but has also been described as highest at the bottom and falling steadily with rising cell position (95, 574) estimates of cell cycle time (T_c) have varied between about 16 and 27 h (98 244, 427, 542).

We have previously described the differences in cytokinetic organisation o the crypts at different sites in the rat colon (689), and this report extends our description of similar variation in the colon of the mouse (589, 685), which may be sufficient to account for some of the inconsistencies in the literature. We have used simple morphometry, the stathmokinetic technique, and autoradiographic methods including the fraction of labelled mitoses (FLM) experiment, at four representative sites in the mouse colon, namely (i) the descending colon (30 percent of the distance from the anus to the ileocaeca valve), (ii) the transverse colon (60 percent of the distance from the anus), (iii) the ascending colon (90 percent of the distance from the anus), and (iv) the caecum (at the junction of the distal and middle thirds of the caecoappendix).

1.4.2 MATERIALS AND METHODS

Male A2G mice 12 weeks old, weighing between 20 and 25 g and conventionally managed were used throughout. They were fed on 14 percent rat cake

(dried milk) and had unlimited access to tap water. In order to minimise the effect on proliferative indices of circadian variation (93, 287, 652) all experiments were begun at 0900 h.

1.4.2.1 MITOTIC AND LABELLING INDICES

Four animals were given ^{3}HTdR of specific activity 5 Ci per mMole by intraperitoneal injection at a dosage of 1 μCi per g body weight. After one hour they were killed by cervical dislocation and their colons removed in continuity with the caecoappendix. The colon was opened along its length, cleaned, and pinned mucosal surface uppermost to a cork board. After fixation for 6 h in Carnoy's solution, transected segments of bowel were taken from the four sites described, and processed through to paraffin wax in the usual way. Autoradiographs were prepared on 3 μm serial sections using a dipping technique, and were exposed for two weeks.

One hundred perfect axially sectioned crypts were located in each sample and the left-hand crypt columns as they presented were analysed. The total number of cells in the column was counted and the positions of mitoses and labelled cells were noted. For each of the selected sites within the bowel, overall mitotic (I_m) and labelling (I_s) index distribution curves were produced by combining the data from the 4 animals and expressing them in terms of a crypt column whose length was the mean of all crypt columns counted at each site (82, 818).

1.4.2.2 STATHMOKINETIC EXPERIMENT

Eleven animals were given an intraperitoneal injection of 1 mg per kg body weight of vincristine sulphate and killed at 15 minute intervals thereafter. The colons were fixed in Carnoy's solution and samples taken from the same 4 sites as before. Serial 3 μm sections were prepared and stained with Harris's haemotoxylin. Again the left sides of 100 axially sectioned crypts were studied, the number of cells per column and the positions of arrested metaphases being recorded. That the stathmokinetic agent was effective in material taken 30 min and longer after administration was confirmed by the absence of any post-metaphase mitotic figures.

In 4 animals from this group the numbers of cells appearing in transverse sections of crypts (the column count) were calculated from 50 such sections at each site in each animal. From the same sections estimates were also made of the correction factors (Tannock's factor) which must be applied to mitotic indices observed in axial sections to compensate for the displacement of mitotic figures towards the lumen of the crypt (702).

1.4.2.3 FLM EXPERIMENT

Thirty-six mice were given ^{3}HTdR by intraperitoneal injection at a dosage of 1 μCi per g body weight, and one animal was killed at hourly intervals for 24 h and thereafter at two-hourly intervals until 48 h. Autoradiographs were prepared as before for each of the four sites in the bowel. The cells in the crypts were divided into groups of 4 cell positions starting at the base of the crypt, and in each group at least 20 mitoses were examined for the presence of label. Thus an FLM curve was constructed for each of the cell position groups, and the phase durations and their variability were estimated (251). An FLM curve for the crypt as a whole was derived by calculating the mean proportion of mitoses labelled, weighted according to the distribution of mitoses within the crypt.

Table 1.4.1 Morphometric and mitotic index data for the whole crypt

	Descending colon	Transverse colon	Ascending colon	Caecum
Crypt column length (cells)	31.1	34.7	19.3	25.3
Number of columns per crypt	22.5	19.6	16.2	25.0
Total number of cells per crypt	700	680	310	630
Observed mitotic index (%)	2.15	1.58	2.04	1.79
Tannock's factor	0.62	0.53	0.57	0.61
Corrected mitotic index (%)	1.33	0.84	1.16	1.09
Birth rate (cells/1000 cells/h)	26	13	16	18
Mitotic duration t_m(h)	0.5	0.7	0.7	0.6

1.4.3 RESULTS

The mean crypt length and the mean column count for each of the 4 sites in the bowel are shown in Table 1.4.1. In the descending and transverse colon, crypts are of a similar length, but in the ascending colon they are much shorter; in the caecum they are of intermediate length, though of a greater circumference. An approximate estimate of the total number of cells per crypt has been found from the product of these two measurements; in the ascending colon there are about half as many cells per crypt as in the other sites.

The data from the stathmokinetic experiment treating the crypt as a whole are presented in Figure 1.4.1; the solid lines have been fitted by least squares to the points from 30 min onward when we are sure that vincristine is fully effective. If the age distribution is known the slopes of these lines can be used to give estimates of k_B, the cell birth rate (see Section 1.4.5.1). The mitotic duration, t_m, can be found, albeit imprecisely, from the ratio of the mitotic index at time zero, I_m, to k_B, and is given in Table 1.4.1 along with the values

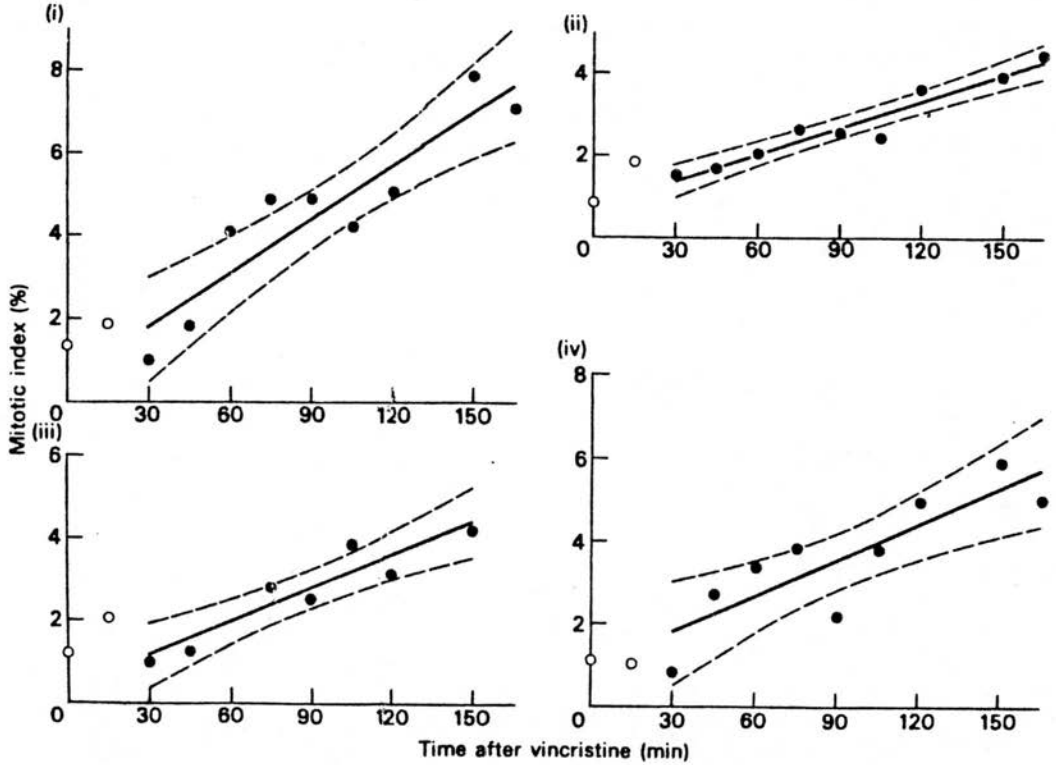

Figure 1.4.1 Mitotic accumulation data for the whole crypt after administration of vincristine, showing fitted lines and 95% confidence limits. Figures (i)–(iv) correspond to the sites in the bowel described in Section 1.4.1

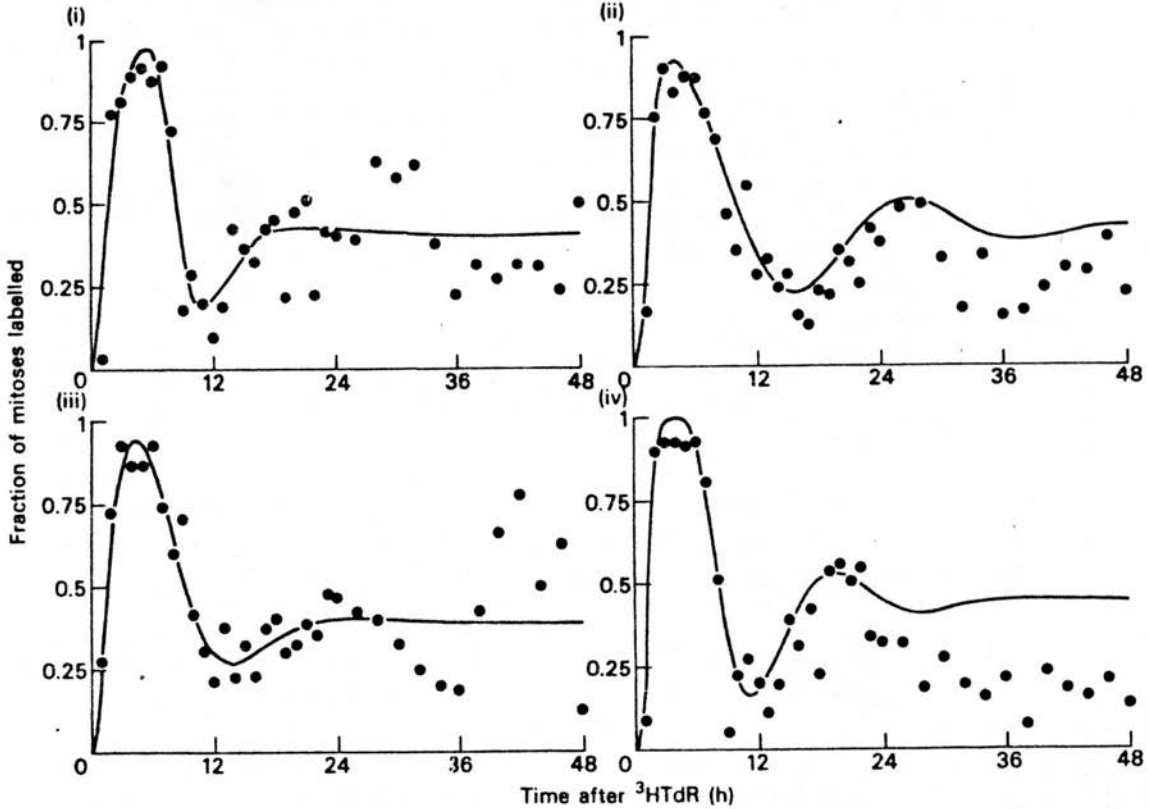

Figure 1.4.2 FLM data for the whole crypt, showing fitted curves. Figures (i)–(iv) correspond to the sites in the bowel described in Section 1.4.1

of these other variables. I_m is presented as it was observed and as it has been corrected by Tannock's factor; the indices in Figure 1.4.1 have all been corrected.

The results of the FLM experiment for the crypts as a whole, and the curves fitted (see Section 1.4.5.2) are shown in Figure 1.4.2. The estimates of the phase durations and the coefficient of variation of T_c are detailed in Table 1.4.2.

Table 1.4.2 Autoradiographic data for the whole crypt

	Descending colon	Transverse colon	Ascending colon	Caecum
t_{G_1} (h)	7.0	10.3	9.4	6.7
t_s (h)	6.2	8.8	7.4	6.8
t_{G_2} (h)	1.8	1.4	1.4	1.2
Cell cycle time T_c (h)	15.5	21.2	18.9	15.3
Coefficient of variation of T_c (%)	43	27	45	30
Theoretical labelling index (%)	43	45	44	48
Observed labelling index (%)	11.7	12.1	12.3	13.5
Growth fraction (%)	27	27	28	28
Total number of proliferating cells per crypt	190	180	90	180
Birth rate (cells/1000 cells/h)	21	14	19	20

The quality of fit is poor for later times, but estimates are probably reasonably accurate since the fit over the first 24 h is adequate. Cell cycle times vary from 15.5 h in the descending colon to 21.2 h in the transverse colon. From the age distribution and the cell cycle parameters a theoretical value for the labelling index if all cells were proliferating may be found, and the proportion of this which is actually observed is an estimate of the growth fraction (see Section 1.4.5.3). Taking this in conjunction with the total number of cells per crypt we can estimate the number of proliferating cells, and from the growth fraction and the cell cycle time we can obtain an estimate of the birth rate independent of that given in Table 1.4.1 (see Section 1.4.5.1). Values for all these variables are given in Table 1.4.2.

By treating each cell position in the crypt separately and performing a total of 110 regression analyses we may calculate the birth rate at each cell position in

Table 1.4.3 Data from mitotic indices at each cell position

	Descending colon	Transverse colon	Ascending colon	Caecum
Cumulative mitotic index (cells/column)	0.36	0.28	0.25	0.28
Cumulative birth rate (cells/column/h)	0.93	0.44	0.36	0.43
Mitotic duration t_m(h)	0.39	0.64	0.69	0.65
Crypt cell production rate (cells/h)	20.9	8.6	5.8	10.8
Cut-off position (cells from crypt base)	8.9	16.7	7.2	8.4

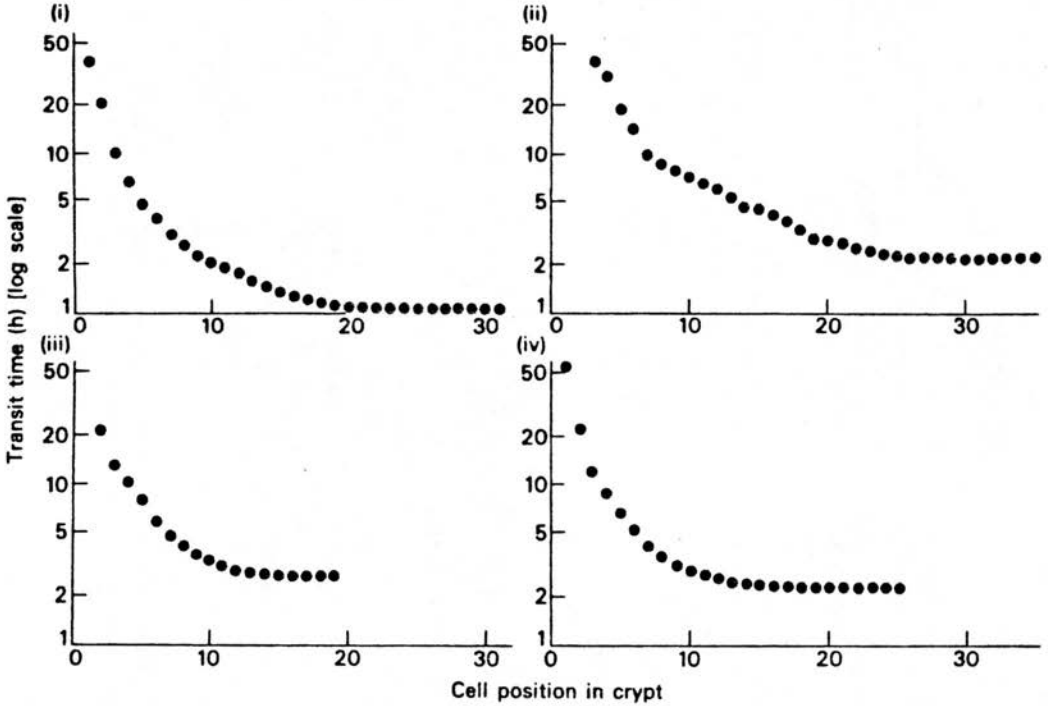

Figure 1.4.3 Transit times from one cell position to the next above. Figures (i)–(iv) correspond to the sites in the bowel described in Section 1.4.1

the four sites. Table 1.4.3 shows the resulting cumulative birth rate or migration rate at the top of the crypt. By dividing this into the cumulative mitotic index an estimate of the mitotic duration may be obtained, which is probably better than the one previously found (589). We may also calculate, from the cumulative birth rate and the column count, the rate of cell production by the crypt; the crypts of the descending colon are producing about 21 cells per hour, some 3 or 4 times more than the short crypts of the ascending colon.

Estimates of the average cell position at which a cell last undergoes a mitosis which gives rise to two proliferating daughter cells (the cut-off position (84)) may also be calculated from the birth rates at each cell position. This involves the derivation of the transit time from a particular cell position to the one above (see Section 1.4.5.4) and these times are shown in Figure 1.4.3. The estimates of the cut-off positions given in Table 1.4.3 suggest that the growth control mechanisms in the transverse colon are rather different from those in the other sites.

Figure 1.4.4 shows the labelling index distribution diagrams for each of the four sites. In the descending colon highest values are found at the bottom of the crypt and there is a steady fall towards the top. In the ascending colon, however, maximum I_s occurs in the middle of the crypt; the transverse colon shows an intermediate pattern, and in the caecum highest values, which are greater than at other sites, are again near the bottom of the crypt.

The cell cycle times derived from the 18 FLM curves resulting from treating each group of 4 proliferative cells separately are given in Table 1.4.4. There are

Table 1.4.4 Cell cycle times (h) for different parts of the crypt

Cell position	Descending colon	Transverse colon	Ascending colon	Caecum
1–4	15.3	22.0	20.6	15.3
5–8	14.9	21.7	18.2	15.5
9–12	15.8	22.0	20.4	16.0
13–16	16.3	20.9	20.0	12.9
17–20	15.4	20.1		

no obvious differences in T_c between different cell position groups within the crypt at any site. From the observed labelling indices of Figure 1.4.4 and the cell cycle phase durations at any cell position we can calculate how the growth fraction (I_p) varies in the crypt (see Section 1.4.5.3), and this is shown in Table 1.4.5. Even in the areas of maximum proliferative activity the growth fraction is well below unity.

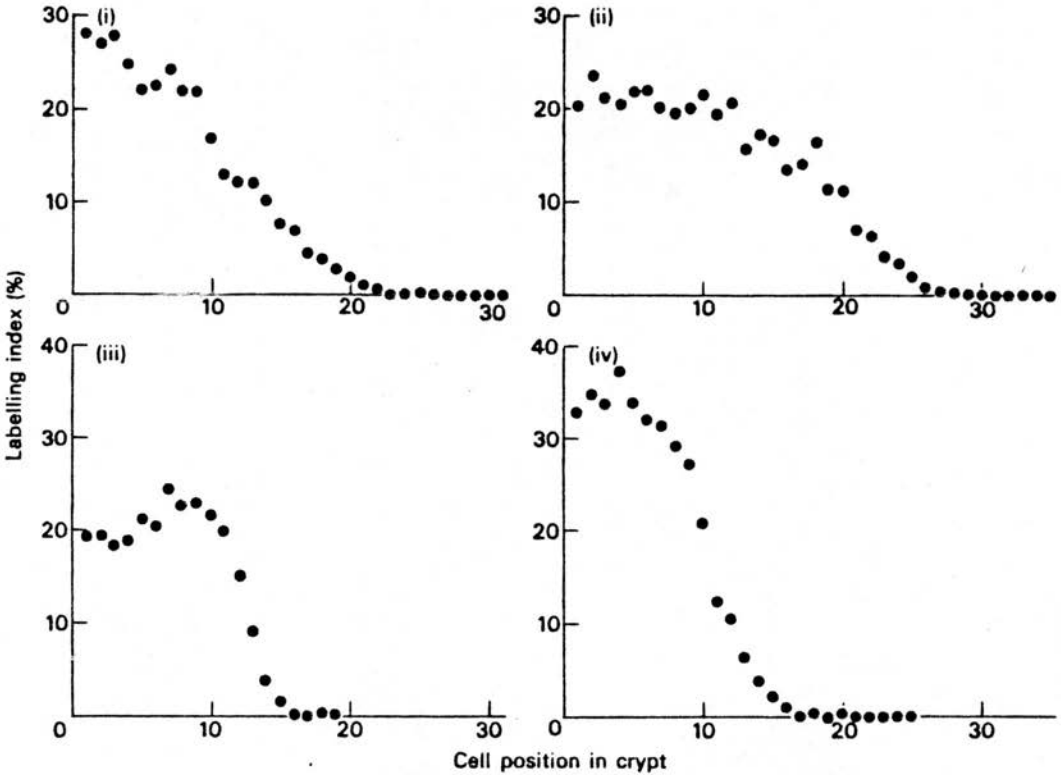

Figure 1.4.4 Labelling index distribution within the crypt. Figures (i)–(iv) correspond to the sites in the bowel described in Section 1.4.1

Table 1.4.5 Growth fraction (%) in different parts of the crypt

Cell Position	Descending colon	Transverse colon	Ascending colon	Caecum
1–4	53	48	44	76
5–8	49	50	54	74
9–12	41	49	53	42
13–16	24	43	10	10
17–20	9	39		

1.4.4 DISCUSSION

We have calculated for the descending colon a mean crypt length of 31 cells, which agrees closely with previous estimates of 29 cells (95) and 28 to 32 cells (574) in two communications where the site in the descending colon from which the material was taken was described in precise anatomical terms. In the transverse colon we have found that the mean crypt length is slightly greater, whereas in the ascending colon it is only 19 cells, close to the value of 20 cells obtained in material from both ascending and sigmoid colon (427), though it is not known whether these two sites were morphologically similar. Our approximate estimates of the total number of cells per crypt show that the ascending colon with 310 cells has less than half the population of the other sites investigated. This number is close to the 358 cells calculated from whole crypt squashes (378) in material whose precise site of origin was not given, but which has the type of distribution of labelled cells which we and others (427, 472) have observed in ascending colon. The distribution of labelled nuclei in the crypts of the descending colon is quite different, with labelling most intense at the bottom of the crypt and falling steadily with rising cell position. This is the sort of pattern previously described in studies on the descending colon (95, 574). In the transverse colon there is an intermediate pattern of distribution of labelled cells.

From the analysis of FLM data we have derived estimates of T_c for the whole crypt at each of the sites in the bowel: the mean values of 15.5 h in descending colon, 21.2 h in transverse colon, 18.9 h in ascending colon and 15.3 h in caecum lead to estimates of cell birth rate similar to those obtained from analysis of the stathmokinetic experiment, after taking the growth fraction and the age distribution into account. Other cell cycle time estimates based on FLM data are to be found in the literature: 19 h (707) and 23 h (98) in descending colon, 16 h in ascending colon (427). We have analysed FLM curves for separate cell position groups within the crypts, and in none of the sites have we demonstrated any differences in T_c within the crypt, though cell migration during the experiment could be masking these.

It has been calculated that between 35 percent and 52 percent of colonic

crypt cells are in the 'proliferative zone' (378) while the 'proliferative compartment size' has been estimated as 37 percent of the total crypt cell population (281). We have estimated I_p for the whole crypt from labelling index data and the cell cycle parameters. The values are virtually the same at all sites, just under 30 percent, though lower than those cited because within that zone of the crypt broadly termed the proliferative compartment I_p is, as we have demonstrated, by no means 100 percent. In absolute terms the crypts of the ascending colon contain about 100 proliferating cells, whereas in the other sites the figure is about 200. However, it is the transverse colon which stands out as being different in terms of the average position at which cells begin their final cycle, where it is twice as far up the crypt as at the other sites.

There are therefore many differences in the kinetic organisation of the crypts at different sites in the mouse colon. Similar differences occur in the large bowel of the rat (688), and, while the two species do not exhibit quite the same pattern of variability, it should be remembered that we have chosen to sample our tissues from anatomically well defined regions rather than from morphologically or functionally equivalent sites.

1.4.5 METHODS OF ANALYSING CELL KINETIC DATA FROM THE INTESTINAL CRYPT

Many of the variables whose values have been derived in this description of cell proliferation in the mouse large bowel are merely the product or the ratio of other variables which have been observed directly; these present no problem, save that it is worth remembering that errors present in the original data are magnified in quantities derived from them, even if the relationship used to calculate them is exact. Usually this effect is compounded by the necessity to make assumptions about the system under consideration before being able to calculate what we may regard as its most useful descriptors. We may be tacitly ignoring known complications such as circadian rhythm, variability in cell cycle phases, or experimental artifacts, or we may be making certain assumptions with regard to the age distribution of cells or their mode of migration. Some of the methods we have used are worthy of further explanation.

1.4.5.1 STATHMOKINETIC EXPERIMENTS

The stathmokinetic experiment has frequently been employed rather uncritically, but carefully used it can give good estimates of the cell birth rate (815). It generally provides only poor estimates of the mitotic duration, and unless the age distribution of cells is known, good estimates of the turnover time cannot be obtained.

For the crypt as a whole we have assumed a rectangular age distribution for proliferating cells, and the values we give for the birth rate are the slopes of the lines relating mitotic index to time after administration of vincristine. For the individual cell positions we have assumed exponential age distributions; this is a good approximation over the most highly proliferative part of the crypt, and the fact that it is far from true near the top of the crypt does not greatly affect the estimate of cumulative birth rate.

We have estimated so many values for this report that to give standard errors of all the estimates would be cumbersome, but it is important to have some idea of the precision of the principal analytical methods. Assuming that the mitotic indices on which we base our calculations are correct, which implies among other things that Tannock's factor exactly compensates for the preferential counting of mitoses, we may expect the standard error of the birth rate to be about 15 percent of the estimate itself; the standard error of the cumulative birth rate is about half this. In fact, as we have indicated, such correction factors as Tannock's and the one for birth rate given below are also subject to uncertainty. By using them we increase the imprecision of our calculated values, but we gain greatly in accuracy.

We have estimated birth rates from autoradiographic data which compare well with those estimated from the stathmokinetic experiment. We shall discuss later the FLM method of obtaining an estimate of the cell cycle time (Section 1.4.5.2) and how the growth fraction should be calculated (Section 1.4.5.3), but even if these quantities are known it is not as straightforward as it seems to estimate the birth rate. For a rectangular age distribution, for instance, the relationship $k_B = I_p/T_c$ has been used (120), but it can be shown (29) that this leads to an underestimate of k_B and that a better estimate is found by dividing this estimate by $1 - CV^2$ where CV is the coefficient of variation of the cell cycle time expressed as a proportion. This correction factor must also be borne in mind when estimates of turnover time or cell cycle time are made from stathmokinetic data.

1.4.5.2 FLM EXPERIMENTS

Most of the methods of analysing FLM experiments in current use make broadly similar assumptions and give comparable estimates of the cell cycle phase durations (301). We have shown in the previous section how important it is to obtain and use estimates of the coefficient of variation of the cell cycle time. It is also important to have some idea of the standard errors of the estimates of phase times. The method we use (251) gives standard errors, and those for the cell cycle time in the 22 FLM curves we analysed averaged one hour. We have already stated (6) that we regard these standard errors as being too low by a

factor of between 2 and 3 in the small bowel, and can only emphasise this opinion in the light of the poorer fits we obtain in the colon.

Another point which is sometimes overlooked by using the simplistic terminology 'the cell cycle time at cell position x' is that by the end of an FLM experiment lasting 48 h, a cell which began as low as position 8 could have migrated right out of the crypt. Thus it is not clear what precisely it is that such an FLM is estimating, and it may be largely because of migration that no differences in cell cycle times at different positions were apparent. A comprehensive model of the crypt is required so that estimates of 'the cell cycle time of a cell born at position x' may be found.

1.4.5.3 ESTIMATING THE GROWTH FRACTION

Estimates of the growth fraction may be made by calculating what proportion the observed labelling index is of a theoretical index which would occur if all cells were cycling. To calculate this latter quantity it is necessary to know the durations of the phases of the cell cycle and the age distribution of cycling cells. However, the usual formulae (120):

$$I_s^{\text{theoret}} = \tau_s/T_c$$

for a rectangular age distribution and

$$I_s^{\text{theoret}} = \{1 - \exp(-0.693\ \tau_s/T_c)\} \exp\{0.693\ (1 - \tau_{G_1}/T_c)\}$$

for an exponential age distribution do not take into account variability in phase durations, and can lead to quite erroneous estimates (27). Figure 1.4.5

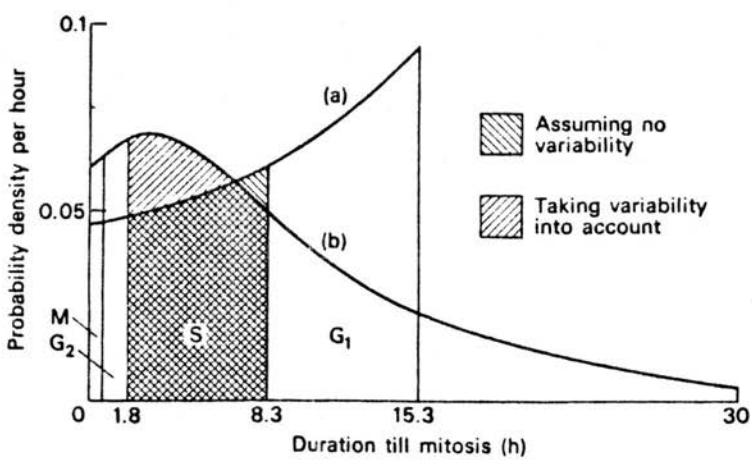

Figure 1.4.5 Distribution of times remaining till the end of mitosis for an exponential age distribution with $\tau_{G_1} = 7.0$ h, $\tau_s = 6.5$ h, $\tau_{G_2} = 1.2$ h and $\tau_m = 0.6$ h assuming (a) a constant cell cycle time and (b) a CV of T_c of 51%

illustrates the case of the bottom 4 cell positions of the caecum. Straightforward application of the formula for an exponential age distribution yields a value for $I_s^{theoret}$ of 0.37, and since the labelling index is 0.34 (see Figure 1.4.4) a growth fraction of 92 percent would be obtained, implying that virtually all cells in this region were proliferating. However, the coefficient of variation of the cell cycle time is 51 percent, and calculation of the area corresponding to labelled cells, which lies under the correct distribution of times remaining until the end of mitosis, gives $I_s^{theoret}$ as 0.45, and hence a considerably lower growth fraction of 76 percent, as shown in Table 1.4.5. When there is little variability in cell cycle time the usual formulae are adequate, but when the coefficient of variation is greater than about 25 percent it is advisable to take it into account. Either overestimates or underestimates of growth fraction may result from ignoring the variability, depending on the relative position in the cycle of the S-phase.

1.4.5.4 ESTIMATING THE CUT-OFF POSITION

Different measurements have been employed to quantify the size of the 'proliferative compartment' (120) in the crypt. In our previous reports on the cell kinetics of the small bowel (814) and the large bowel (689) we have calculated the cell position at which the labelling index falls to half of its maximum value; this was done by projecting individual crypts onto one of average length (82, 818). Another possible method is to calculate the mean position over a number of crypts of the highest labelled cell (287). Both these methods are less than completely satisfactory because the cell position they define will be moved up the crypt not only by a real increase in the extent of the proliferation zone caused by more transit divisions, but also by a decrease in cell cycle time or an increase in growth fraction within the proliferation compartment (28). It is possible to calculate the number of transit divisions from stathmokinetic and FLM data (814), but we believe that the best single descriptive measurement is the cut-off position (84): the average position at which a cell enters its final cycle. We have described a simple calculation (28) based on working out from transit times where a cell at the position of half maximum mitotic index near the end of a stathmokinetic experiment would have been a certain time previously. This calculation does not take into account all of the aspects of the 'slow cut-off model' (84) but we feel that it is adequate to draw clear distinctions between crypts with different characteristics.

ACKNOWLEDGEMENTS

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Mrs E Wallace and Mrs M Hughes for preparing the histological sections.

David Appleton

J. Anat. (1975), **119**, 1, pp. 105–121
With 12 *figures*
Printed in Great Britain

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The effect of starvation and refeeding on cell population kinetics in the rat small bowel mucosa

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(*Accepted* 14 *August* 1974)

INTRODUCTION

In recent years there has been increasing interest in the control of cell proliferation, especially in renewal systems such as the small intestinal mucosa. While studies of cell kinetics under normal physiological conditions have proved valuable (Cairnie, Lamerton & Steele, 1965*a*, *b*), more information about the control mechanisms has come from experiments designed to provoke the renewal systems into making a series of compensatory adjustments. One such stimulus which has received a good deal of attention is starvation.

The morphological sequelae of starvation in the rat have been described by Brown, Levine & Lipkin (1963) and comprise villous tip destruction, with basal fissuring and shortening of villi. A decrease in the villous cell population was also suspected by Stevens Hooper & Blair (1958) whose studies further demonstrated a reduction in crypt cell population in rats starved for 5 days. Altmann (1972) confirmed the reduction in crypt and villous size after 5 days' starvation, while Hopper, Wannamacher & McGovern (1968) reported a 33% decrease in crypt population in the rat after 10 days' starvation. On a broader front, Clarke (1972) found a small reduction in the total number of crypts in rats starved for 5 days.

Accompanying this hypoplastic response there were considerable changes in crypt cell kinetics: Weibecke, Heybowitz, Lohrs & Eder (1969) showed a reduction in labelling and mitotic indices in mice starved for 4 days, due to an increase in the duration of the cell cycle; Rose, Hopper & Wannamacher (1971) demonstrated only a small increase in the duration of DNA synthesis in starved rats, but Altmann (1972) reported a prolongation of turnover time, in similar circumstances.

These starvation-induced hypoproliferative responses in the small bowel mucosa have been wholly attributed to the increase in cell cycle time (Hopper, Rose & Wannamacher, 1972), but most workers have overlooked the important role of the *growth fraction*, i.e. the proportion of crypt cells engaged in proliferation. Changes in this parameter, as well as in the cell cycle time, control the rate of crypt cell production, and a reduction in the growth fraction could contribute to the demonstrated reduction in proliferative indices. If this is the case then some proliferating crypt cells may subside into a resting state during starvation and return to activity on the resumption of feeding.

A third possibility arises from the studies of Cameron & Cleffman (1964): when starved chicks were fed the labelling index rose almost immediately, but the mitotic index rose only after an interval of 8 hours; this indicated that cells were arrested in the G_1 phase of the cell cycle, and had to complete DNA synthesis before entering mitosis.

It therefore becomes necessary to differentiate between the relative contributions of the established increase in cell cycle time, and a possible decrease in proliferating population, to the hypoproliferative response to starvation; and also to determine whether or not there is a specific control point in G_1 at which cells become blocked in their progress around the cell cycle. In this paper we consider the effects of starvation and refeeding on proliferative indices, growth fraction and cell cycle time in the rat small bowel.

MATERIALS AND METHODS

Male albino Wistar rats aged 3 months and weighing 150–200 g were used throughout. In the labelling experiments animals received 0·5 μCi per g body weight of tritiated thymidine, ^{3}HTdr (Radiochemical Centre, Amersham, England) by intraperitoneal injection 1 hour before death. The ^{3}HTdr was of specific activity 5 Ci/mM, and the concentration used was 100 μCi per ml. In the stathmokinetic experiments each animal was given 1 mg per kg body weight of vincristine sulphate (Eli Lilly and Co. Ltd.) by intraperitoneal injection. The rats were killed by cervical dislocation. Samples of small bowel were taken from the region immediately below the ligament of Treitz, and may thus be described as upper jejunum. The tissue was fixed in Carnoy's fluid for 6 hours and processed through to paraffin. Transverse sections were cut at a thickness of 3 μm and stained with Harris' haematoxylin.

Autoradiographs were prepared using Kodak AR 10 stripping film and exposed for 28 days; the slides were developed using Kodak D19b and were then fixed, washed and stained through the emulsion with Harris' haematoxylin.

Experiment 1

Rats were starved continuously for 96 hours starting at 0900 hours, but were allowed free access to water. Animals received ^{3}HTdr one hour before death, and one animal was killed every four hours during the experimental period.

Experiment 2

After 96 hours starvation, again starting at 0900 hours, rats were allowed free access to food. After this comparatively short period of starvation the animals responded by immediate resumption of eating. During the first 24 hours after refeeding one animal was killed every two hours, and subsequently every three hours up to 48 hours after refeeding. Animals again received ^{3}HTdr one hour before death.

Experiment 3

Stathmokinetic studies were carried out at 96 hours after starvation, and after 16 hours refeeding of rats previously starved for 96 hours. In an attempt to minimize

the effects of diurnal variation (Sigdestad, Bauman & Lesher, 1969) vincristine was consistently injected at 0900 hours. Animals were killed at intervals of 15 minutes up to 150 minutes after vincristine. In each animal the mitotic index was measured by studying 100 crypt sections as detailed below.

Counting techniques

In each animal the labelling index and/or the mitotic index was obtained by analysing 100 axially sectioned crypts. In each crypt the left hand column of cells was numbered, counting from the bottom upwards to the crypt–villus junction, and the positions in this *crypt column* of labelled or mitotic nuclei were recorded. A cell was regarded as labelled if 5 or more grains were localised over the nucleus, and the criteria for the recognition of the various mitotic stages were those of Clarke (1970). In the animals killed 15 minutes after vincristine injection a few telophases were still apparent; but thereafter only prophases and metaphases were seen and counted.

The data given by these techniques allowed construction of *labelling and mitotic index distribution curves* (Cairnie *et al.* 1965*a*) in which the labelling or mitotic index was plotted as a function of cell position in the crypt. The variation in the height of the crypt columns (Cairnie & Bentley, 1967) was compensated for by analysing the data by an ALGOL computer program run on an IBM 360/67 computer. The details of the method are given elsewhere (Wright, Morley & Appleton, 1972*a*, *b*).

In the crypts two morphometric parameters were measured: the length of the crypt column in terms of cell number was measured in the analysis of the axially sectioned crypts described above; the number of cells in cross sections of crypts is the *column count* (Cairnie, 1967) which was measured by counting 100 crypt cross sections in each animal. The product of the crypt column length and the column count gives an estimate of the *total crypt population* (Wimber & Lamerton, 1963; Cairnie, 1967).

RESULTS AND INTERPRETATION

Morphometric parameters

After starvation the crypt column length had decreased from $33{\cdot}1 \pm 0{\cdot}3$ (S.E.) cells at 4 hours to $29{\cdot}9 \pm 0{\cdot}2$ cells at 96 hours. Fig. 1 shows that the crypt column length decreased slowly over the experimental period. This was accompanied by a small reduction in the column count (Fig. 1) which fell from $20{\cdot}1 \pm 0{\cdot}1$ cells to $19{\cdot}3 \pm 0{\cdot}1$ cells. The total crypt population, calculated from the product of the crypt column length and the column count, showed a reduction from 670 cells to 580 cells after 96 hours' starvation, a 12% reduction (Fig. 1).

With the resumption of feeding, the column count showed a slow return towards control values by 48 hours after refeeding (Fig. 2). The crypt column length, after an initial increase during the first 12 hours of refeeding, fell again over the remainder of the experimental period. The net effect of these changes on the total crypt population is shown in Figure 2: after some variation over the first 24 hours of refeeding, the crypt population was maintained at a constant value slightly below the control value.

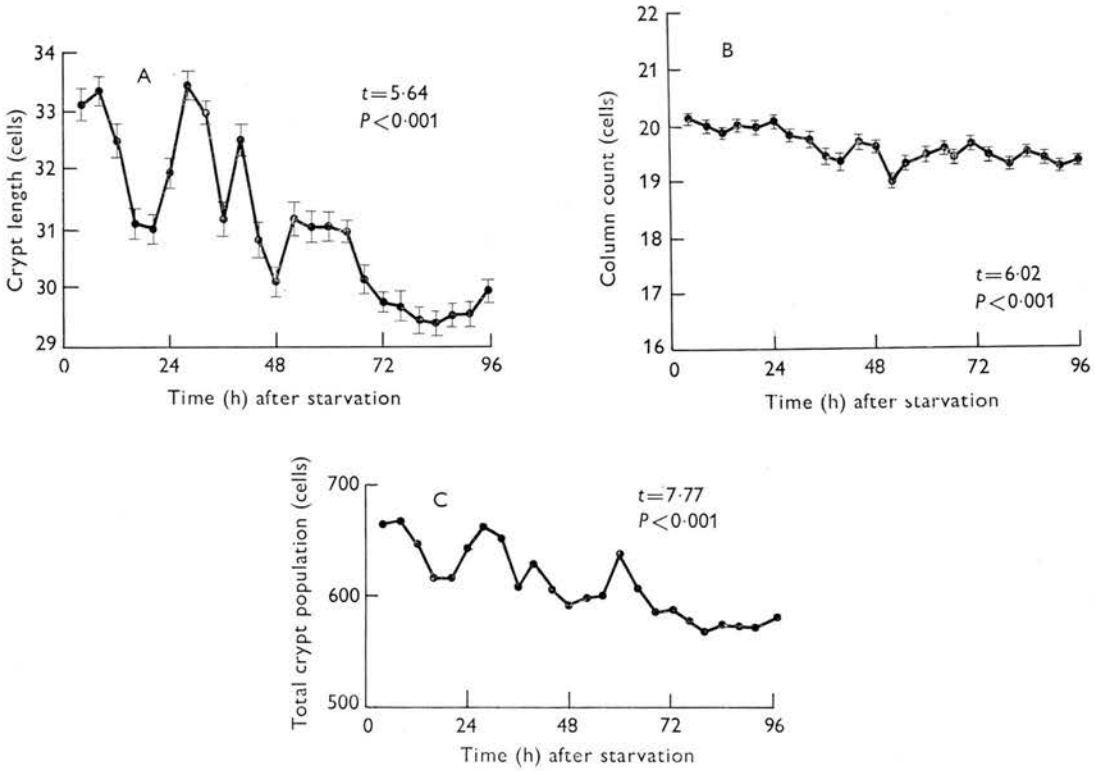

Fig. 1. Changes in crypt column length (±S.E.), column count (±S.E.) and total crypt population during 96 hours' starvation in male rats.

Proliferative indices

The labelling index (Fig. 3) showed a steady fall over the 96 hours of starvation, decreasing from 36·4% to 23·3%. The mitotic index appeared to remain constant over the first 40 hours and then decreased from values above 6·0% to reach 4·5% after 96 hours' starvation.

Upon refeeding the labelling index increased quickly to reach a peak of 34·5% after 12 hours (Fig. 4); this was followed by a small decrease over the next 10 hours before constant values of around 30% were realized for the remainder of the experiment. In the case of the mitotic index a latent period of approximately 6 hours was apparent before the mitotic index began to rise steadily to a peak of 9·6% at 16 hours.

The fall in proliferative indices during the period of starvation was gradual and whatever mechanism was responsible for the fall operated slowly. On the other hand, compensatory changes after refeeding were rapid. While the present data did not admit of definitive interpretation, we may speculate that the first kinetic event in the proliferative response was an increased flow of cells into DNA synthesis, and later into mitosis. It is evident that this postulated sequence could be brought about by the resumption of the progress into S of cells which were originally arrested in G

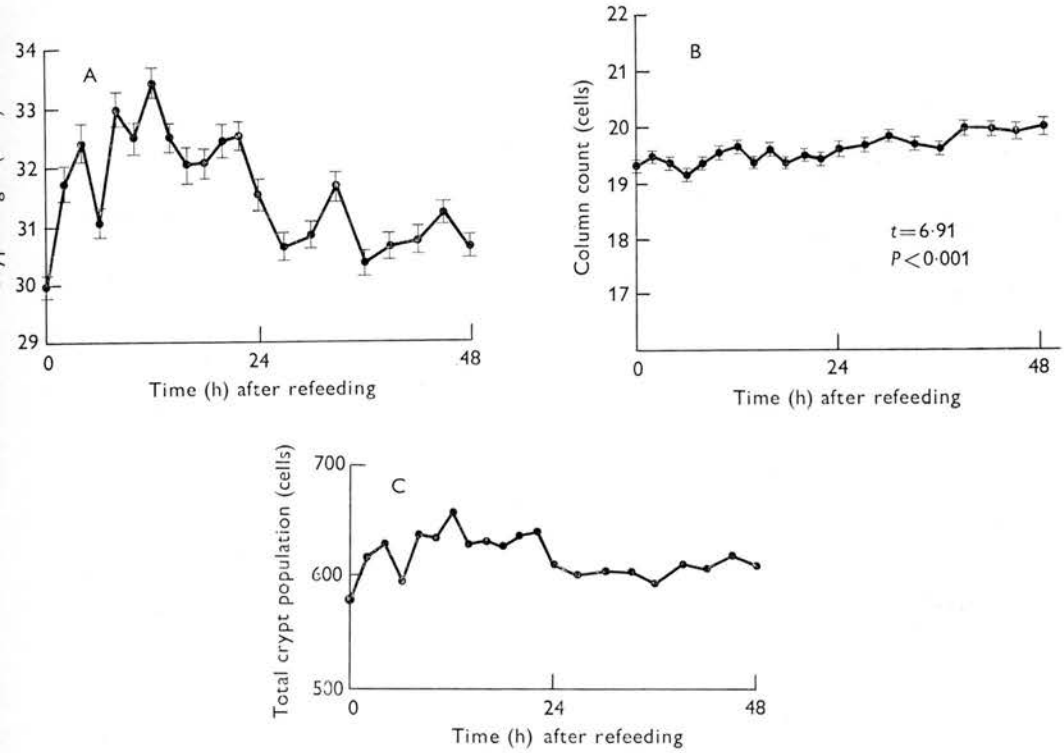

Fig. 2. Changes in crypt column length (±S.E.), column count (±S.E.) and total crypt population with time after refeeding male rats starved for 96 hours.

or in a G_0 compartment, or by an increased G_1 to S flow due to a reduction in the duration of G_1.

Proliferating populations

Fig. 5*a* shows the labelling index distribution curve for the animal killed 4 hours after starvation. Low values were found in the bottom few cell positions, followed by a peak of over 75% in cell positions 5–9. Thereafter a steady decrease took place over the remaining cell positions. By drawing a perpendicular at the cell position of the 50% peak value, an estimate could be obtained for the crypt growth fraction (the fraction of crypt cells actually engaged in the cell cycle). A growth fraction (I_p) calculated for the whole crypt represented the fraction of the crypt population which occupied the proliferative compartment; this was the region over which labelled cells were observed, and may be considered to end at approximately the cell position where the labelling index fell to 50% of its maximum value (Cleaver, 1967). Wright *et al.* (1972*a*) showed good agreement between values calculated by this method and those obtained from FLM curves. In Fig. 5*a* the 50% peak value is at position 17, and the growth fraction for the whole crypt may be taken as the ratio of the proliferative compartment size of 17 cells to the total number of cells in the crypt, i.e. 17/33 (0·51). Labelling index curves were constructed for each

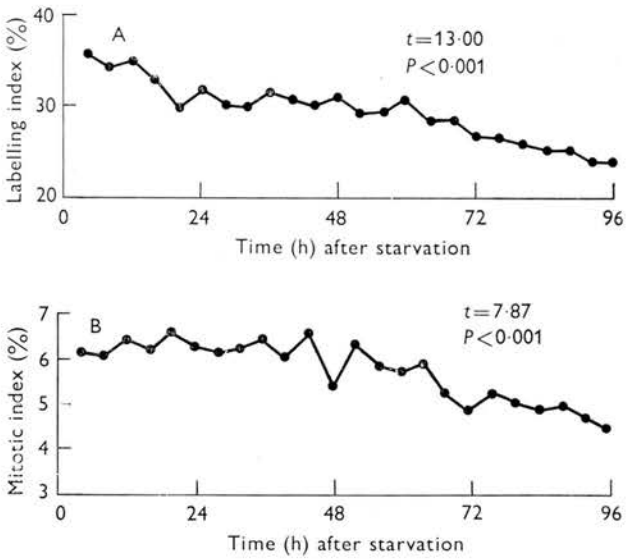

Fig. 3. Changes in labelling and mitotic indices during 96 hours' starvation in male rats.

animal and values of the growth fraction obtained in the same way. The growth fraction as a function of time after starvation is shown in Fig. 6, with the labelling index distribution curve for the animal killed at 96 hours depicted in Fig. 5*b*.

The growth fraction at 96 hours was 0·52, almost the same as that at 4 hours, and it can be seen from Fig. 6 that throughout the duration of the starvation period I_p remained between 0·50 and 0·55. It was concluded that the changes in growth fraction did not contribute to the demonstrated reduction in proliferative indices.

Changes in the growth fraction with time after refeeding are shown in Fig. 7. After a small initial decrease only a slight rise was apparent, with a peak value of 0·55 at 8 hours after refeeding. This was followed by a fall to reach a substantially constant value of around 0·5 during the remainder of the experiment. This increase in I_p during the initial phases of the proliferative response was not significant, and it could be proposed with confidence that such a small increase in the growth fraction could not play an important part in the rise in proliferative indices after refeeding. Labelling index distribution curves are shown for the animals killed at 8 and 16 hours after refeeeding in Figs. 5*c* and 5*d*.

An estimate of the total number of proliferating cells per crypt could be obtained from the product of the growth fraction and the total number of cells in the crypt, and the changes during starvation are shown in Fig. 8. A small decrease from about 350 cells per crypt to 300 occurred over the 96 hours; as the growth fraction remained substantially constant during this period, this reduction in total proliferating cells was a direct reflection of the decrease in crypt population (Fig. 1). Upon refeeding (Fig. 9) an increase to control values occurred initially, due largely to the small increase in growth fraction (Fig. 7) but also partly to an increase in total crypt population. Values then settled at around 300 cells for the remainder of the experiment.

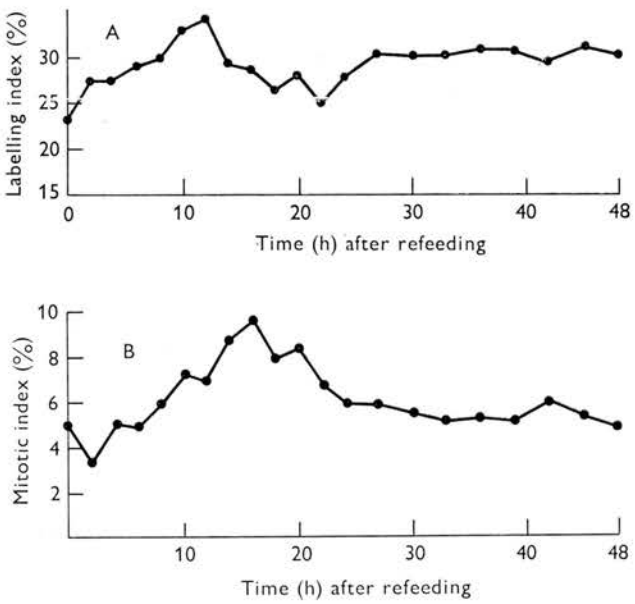

Fig. 4. Changes in labelling and mitotic indices with time after refeeding male rats starved for 96 hours.

Cell cycle parameters

We have shown that considerable decreases in the proliferative indices in the small bowel mucosa can be produced by 96 hours' starvation, followed by rapid rises after refeeding. A hypoplastic response was also evident in the form of decreased numbers of crypt cells. During starvation there was no demonstrable change in growth fraction, and it was therefore necessary to look closely at changes in the cell cycle time.

The mitotic index accumulation line after vincristine injection at 96 hours of starvation is shown in Fig. 10*a*. The readings prior to 30 minutes after vincristine were ignored because of uncertainties concerning the stathmokinetic events during the early stages of the experiment (Clarke, 1971; Pelc, 1971; Wright *et al.* 1972*b*). A good degree of linearity was apparent over the stathmokinetic period.

The slope of the line is a function R of the rate of entry into mitosis and is equal to 5·74 % of cells per hour. For absolute values a further factor should be considered, viz. Tannock's constant (Tannock, 1967; Wright *et al.* 1972*b*) by which R must be multiplied in order to compensate for the spatial arrangement of cells in the crypt. This value in male Wistar rats was 0·62 (Wright *et al.* 1972*b*). Therefore r, the rate of entry of cells into mitosis per proliferating cell of the population, is related to the rate of entry as a percentage by

$$r = \frac{5{\cdot}74}{0{\cdot}52 \times 100} \times 0{\cdot}62 = 0{\cdot}0684. \quad (1)$$

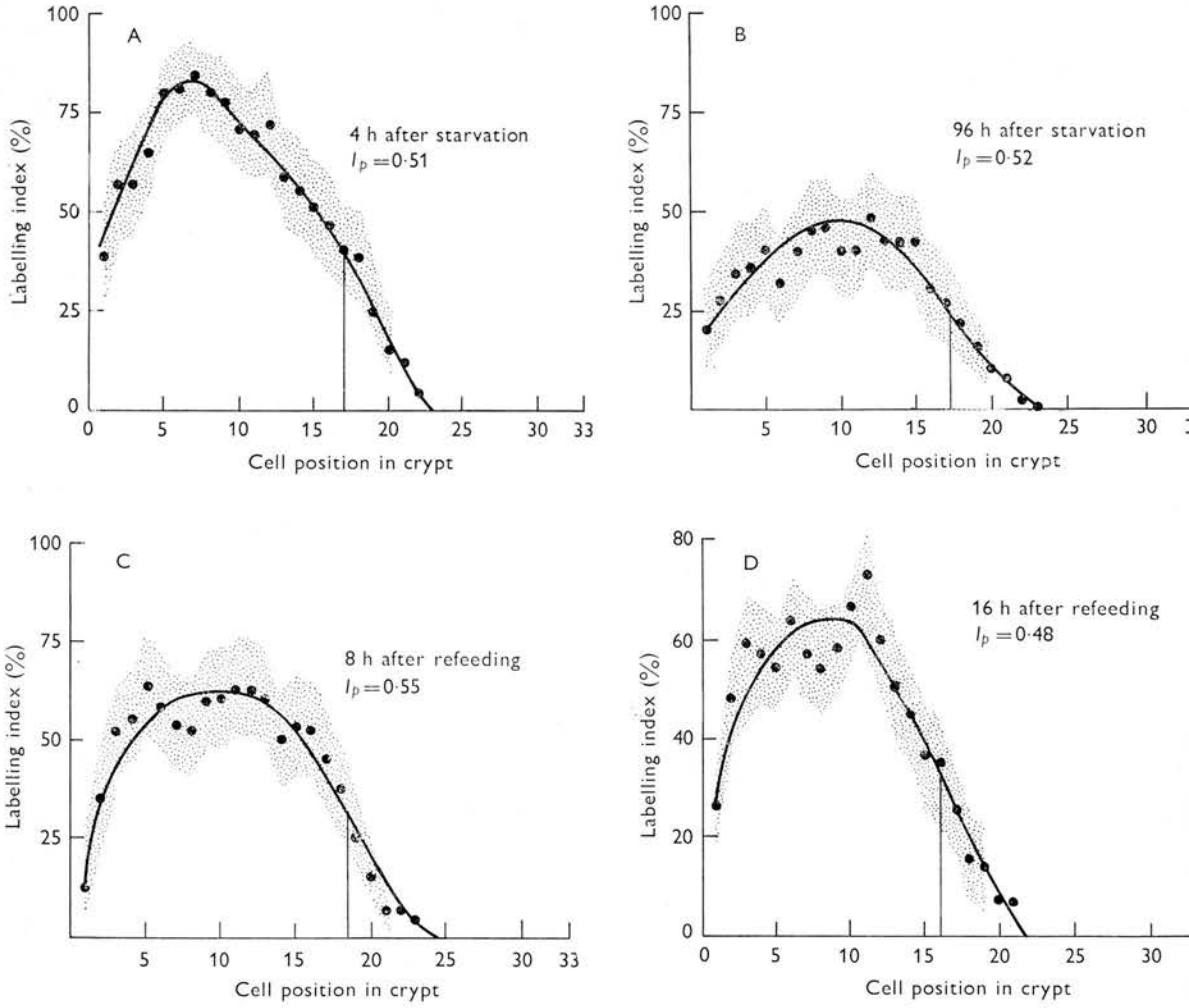

Fig. 5. Labelling index distribution curves (A after 4 hours' starvation, B after 96 hours' starvation, C 8 hours after refeeding, and D 16 hours after refeeding). The shaded areas indicate 95 % confidence limits for the points.

Assuming steady state conditions within the population, the mean cell cycle time (T_c) is the reciprocal of the rate of entry into mitosis or

$$T_c = \frac{1}{r} = \frac{1}{0{\cdot}068} = 14{\cdot}7 \text{ hours}$$

compared with a control value of 10·4 hours (Wright *et al.* 1972*b*); the difference is significant ($P < 0{\cdot}001$), and we concluded that the decrease in proliferative indices evoked by starvation was due entirely to prolongation of the cell cycle time. The mitotic duration (Wright *et al.* 1972*b*) was also prolonged at 1·0 hour, compared with a control value of 0·4 hour.

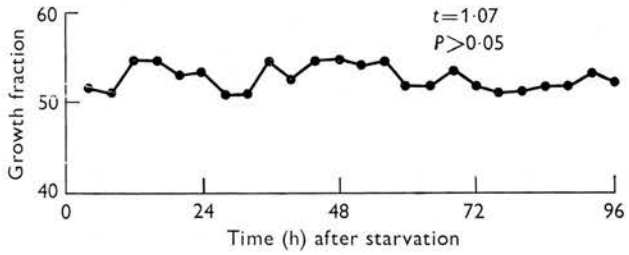

Fig. 6. The growth fraction as measured from the labelling index distribution curve for animals killed during 96 hours' starvation.

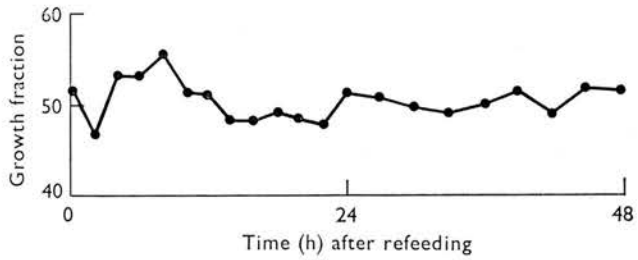

Fig. 7. Growth fraction changes with time after refeeding male rats starved for 96 hours.

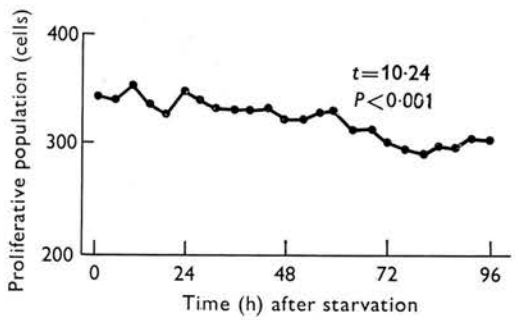

Fig. 8. Total proliferating cells per crypt plotted against time after starvation.

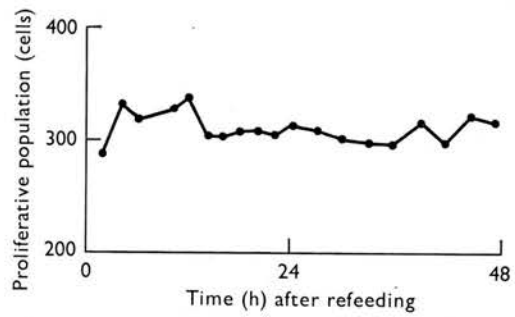

Fig. 9. Changes in total proliferating cells per crypt with time after refeeding male rats starved for 96 hours.

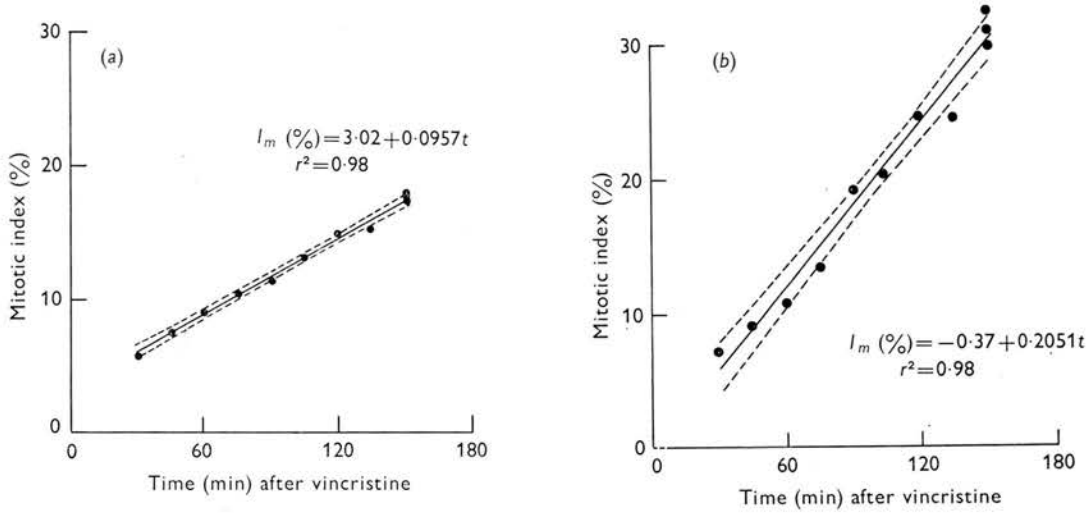

Fig. 10. (*a*) the mitotic accumulation line with time after vincristine injection for the whole crypt column of male rats starved for 96 hours and (*b*) for the whole crypt column of rats after 16 hours' refeeding. The broken lines indicate 95 % confidence limits for the lines.

The mitotic index accumulation graph of 16 hours after refeeding, i.e. at the peak of the mitotic index response, is shown in Fig. 10*b*. Again there is a good degree of linearity. However,the 95 % confidence limits obtained in this experiment were wider than those after 96 hours' starvation (Fig. 10*a*) and in control animals (Wright *et al.* 1972*b*); this could be due to failure to take into account the rate of entry into mitosis in a situation where the mitotic index was itself increasing; in other words, the situation was dynamic with respect to changes in T_c. We return to this point in the discussion. The slope of the line in Fig. 10*b* is significantly greater than that shown in Fig. 10*a* ($P < 0{\cdot}001$).

The cell cycle time, calculated as detailed above, and using the growth fraction measured at 16 hours (0·48), was 6·55 hours. The mitotic duration was also shortened to 0·6 hour. The crude rate of entry into mitosis (R) was 12·3 % of cells per hour. The increase in proliferative indices which occurred after refeeding starved animals was therefore attributable to a reduction in cell cycle time.

The cell cycle parameters measured are summarized in Table 1, together with other parameters measured at the same time intervals.

Flux parameters

Wright *et al.* (1972*b*) have shown that in order to calculate an accurate *cumulative birth rate curve* the mitotic duration must be measured at each cell position. Mitotic accumulation graphs were plotted for each individual cell position in the same way as for the whole crypt column, and the mitotic duration calculated as before (Wright *et al.* 1972*b*).

Table 1. *Changes in cell kinetics in the crypts of Lieberkühn of the rat after* 96 *hours' starvation, and* 16 *hours after subsequent refeeding*

	Crypt population (cells)	Growth fraction	Total proliferating cells per crypt	Labelling index (%)	Mitotic index (%)
Control	670	0·51	340	36·0	6·2
96 h starvation	580	0·52	301	23·3	4·5
16 h refeeding	630	0·48	310	28·5	9·7

	Crude rate of entry into mitosis (%/h)	Cell cycle time (h)	Mitotic duration (h)	Migration rate (cell positions/h)	Cell production rate (cells/crypt/h)
Control	11·2	10·4	0·40	1·8	39
96 h starvation	5·7	14·7	1·0	1·0	19
16 h refeeding	12·3	6·5	0·60	1·9	38

	Transit time (proliferative compartment) (h)	Transit time (maturation compartment) (h)	Transit time (proliferative+ maturation compartments) (h)
Control	26	8	34
96 h starvation	83	15	98
16 h refeeding	33	9	42

Cell cycle and flux parameters for control animals are taken from Wright, Aldewachi, Appleton & Watson, in preparation.

The *birth rate* (k_b) at each cell position was then calculated, assuming steady state conditions, from the equation

$$k_b = \frac{I_m}{t_m}. \tag{2}$$

The values for each cell position were added, and the resulting cumulative birth rate was plotted as a function of cell position in the crypt column. Cumulative birth rate curves are shown for rats after 96 hours' starvation (Fig. 11*a*) and after 16 hours' refeeding (Fig. 11*b*). A higher limiting value was apparent in the refed animals.

The birth rate is equal to the rate of cell production (cells/crypt/hour) at each cell position; consequently the maximum value attained is the rate of cell production for the whole crypt column. An estimate of the *crypt cell production rate* may be obtained from the product of the cell production rate per crypt column and the number of columns in the crypt, the column count.

After 96 hours' starvation the crypt cell production rate was reduced to 19 cells/crypt/hour, compared with a value for control, normally fed rats of 39 cells/crypt/hour (see Table 1). Sixteen hours after refeeding the starved animals, the crypt cell production rate was a near normal at 38 cells/crypt/hour. Assuming that movement of cells within the crypt resulted only from crypt cell proliferation

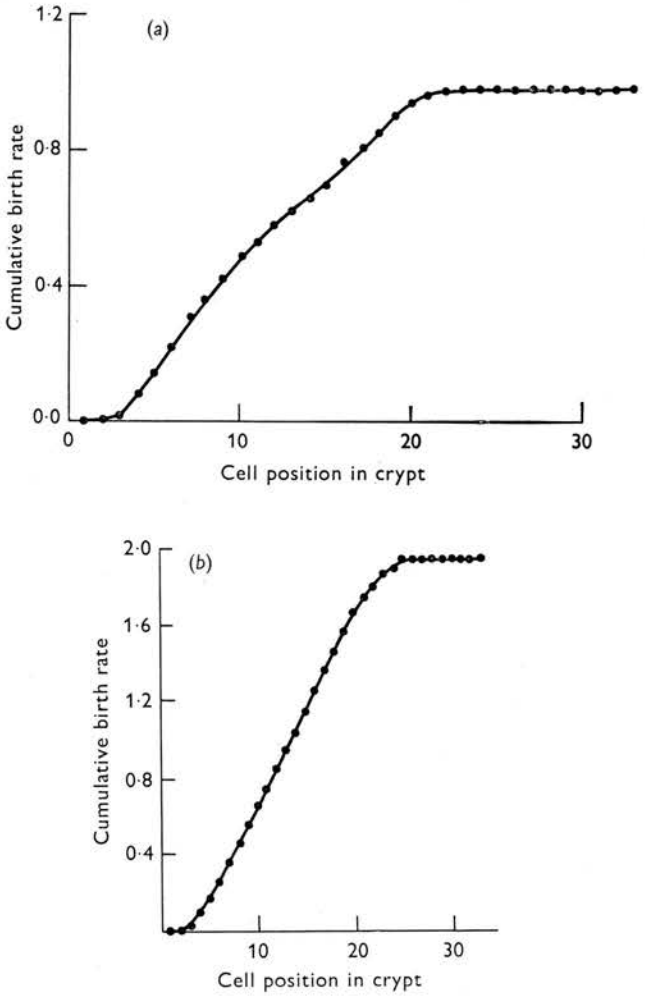

Fig. 11. Cumulative birth rate curves for (*a*) rats after 96 hours' starvation, and (*b*) rats after 16 hours' refeeding.

(Cairnie, Lamerton & Steele, 1965*b*) then the maximum birth rate attained was also equal to the migration rate at the top of the proliferative compartment. This was 1·0 cell positions/hour after 96 hours' starvation, increasing to 1·9 cell positions/hour 16 hours after refeeding. The value for control, normally fed rats was 1·8 cell positions/hour.

The size of the proliferative and maturation compartments in terms of the crypt column was measured from the labelling index distribution curves. The growth fraction, and hence the relative size of the proliferative compartment, remained substantially constant throughout both starvation and refeeding, but there was an absolute decrease in proliferative compartment population because of the reduction

in crypt size. The *transit times* through these proliferative and maturation compartments could be calculated from the cumulative birth rate curves, the reciprocal of the cumulative birth rate at each position being equal to the time taken to traverse that position. Adding these reciprocals over the relevant portion of the crypt gave transit times. In the rats starved for 96 hours, the transit time through the proliferative compartment was increased to 83 hours compared with our control values of 26 hours, and maturation compartment transit time was also increased from a control value of 8 hours to a value of 15 hours after 96 hours' starvation. In the refed animals on the other hand, transit times were reduced to 33 hours in the proliferative compartment, and 9 hours in the maturation compartment. These values are summarized in Table 1.

DISCUSSION

This study has shown that during starvation in the rat there is a reduction in crypt population. This hypoplastic response confirms the earlier findings of Stevens Hooper & Blair (1958), Hopper *et al.* (1968) and Altmann (1972). We have also demonstrated a reduction in labelling and mitotic indices during starvation, confirming the findings of Weibecke *et al.* (1969) in the mouse; however, Hopper *et al.* (1968) were unable to show a decrease in proliferative indices even after 9 days' starvation in the rat. On the other hand Stevens Hooper & Blair (1958) reported a decrease in the number of mitoses per 'crypt section', but this result might be expected on account of the decrease in crypt population.

A reduction in proliferative indices could in principle be caused by a decrease in the growth fraction, that is in the proportion of proliferating cells, or by a decrease in the rate at which cells traverse the cell cycle. We have shown that there is no change in the growth fraction after starvation, and that the reduction in labelling and mitotic indices is due wholly to an increase in cell cycle time. Changes in growth fraction have not hitherto been considered, but we have used the data of Hopper *et al.* (1968) and Rose *et al.* (1971) in the rat, and of Weibecke *et al.* (1969) for the mouse and find no change in the growth fraction as computed from the reported cell cycle parameters and the labelling index. We conclude that changes in growth fraction play no part in the hypoproliferative response evoked in the small bowel mucosa by starvation. This raises the general question of the importance of growth fraction changes in hypoproductive states: upward movement of the 'cut-off position', and hence an increase in growth fraction, has been found during recovery from continuous irradiation (Cairnie, 1967) and in androgen stimulation of the small bowel in the castrate mouse (Wright *et al.* 1972*a*), and this mechanism may augment cell production in hyperproliferative states. Nevertheless, we must not dispense with the growth fraction as a mechanism for reducing cell production, as Wright *et al.* (1972*a*) demonstrated a decrease in growth fraction from 0·65 in control to 0·40 in castrated mice. It is probable that different mechanisms are important in hypoproliferative states in different experimental situations.

Prolongation of the cell cycle time is evidently the mechanism producing the hypoproliferative mucosal state in starvation of the rat, in agreement with the findings of Weibecke *et al.* (1969) in the mouse. We have shown a substantial and significant increase in T_c from 10 hours in controls to 14·7 hours in starved animals. Rose *et al.*

(1971) reported FLM measurements on rats starved for 9 days and, while no real change was found in T_c, a small increase in the duration of the S phase was reported. But this increase was very small, and of questionable importance in the absence of a reliable computer analysis. Furthermore these workers did not report any change in labelling index. However Altmann (1972) has provided some support for the present data by showing an increase in turnover time in rats starved for 7 days. Future work will have to show which phases are responsible for the demonstrated prolongation in T_c; Wiebecke *et al.* (1969) reported increases in all phases, but G_1 was most prolonged.

Upon refeeding the recovery sequence involves a prominent shortening of T_c, from 14·7 to 6·5 hours. Here we have calculated T_c at 16 hours (after refeeding) for comparative purposes only. Fig. 10*b* shows an increased rate of entry into mitosis after refeeding which does not necessarily indicate a reduction in T_c, and may also be explained by an influx of cells from a shortened G_1. We conclude that starvation and subsequent refeeding both involve a modulation of cell cycle time. The small and transient increase in growth fraction noted in the first few hours of refeeding (Fig. 7) is considered to be much less important than the T_c changes in explaining the increased cell production rate. Indeed, at the actual time when cell production rate was measured, i.e. at 16 hours after refeeding, the growth fraction (0·48) was actually lower than that after 96 hours' starvation (0·57).

We have demonstrated an increase in T_c in starvation and a subsequent decrease upon refeeding, but are unable to say with certainty which phase of the cell cycle is predominantly affected. The findings are consistent with a considerable and immediate shortening of t_{g_1}, cells completing DNA synthesis before entering mitosis. Cameron & Cleffman (1964) found a temporal differential between proliferative indices on refeeding starved chicks: such a differential would be consistent with a blockage of cells in G_1, and subsequent release on refeeding. Inspection of the labelling index distribution curve at 96 hours after starvation shows a decrease in the maximum or peak labelling index realized in the proliferative compartment, compared with the peak value after only 4 hours' starvation; also the peak value is again increased after refeeding. Cleaver (1967) noted that, in control animals, the theoretical labelling index as calculated from t_s and T_c is realized in the proliferative compartment, indicating that few, if any, cells are in a resting or G_0 phase within the compartment. Not having measured t_s during starvation and in the initial phases of refeeding we are unable to exclude movement into a G_0 phase within the proliferative compartment after starvation, and later re-entry into the cell cycle with refeeding. Even if this does occur, however, the resting phase here is not a G_0 phase in the strict sense of the term (as applied for example to hepatocytes before entering the proliferative cycle in response to partial hepatectomy), for the labelling index rose immediately after refeeding, with no hint of the pre-replicative period which is common to models of induced DNA synthesis (Stein & Baserga, 1972). We concede that a block in the flow of cells from G_1 to S is possible, and further studies using FLM techniques may settle this question: the changes which have been demonstrated in T_c may be sufficient to account for the observed changes in proliferative indices.

Previous studies on kinetic events after refeeding starved animals have been few,

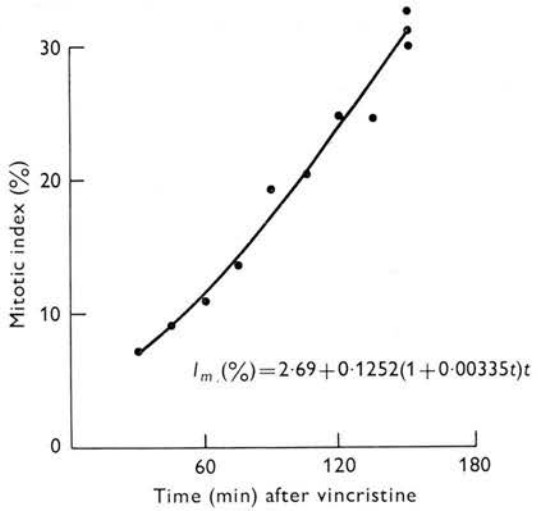

Fig. 12. The data from figure 11(*b*), i.e. the mitotic accumulation line after 16 hours' refeeding, fitted by a quadratic expression.

but Altmann (1972) reported an increase in mitotic index after refeeding rats starved for 7 days; there was also a decrease in turnover time within 24 hours, in accord with the present observations. Altmann failed to find any increase in crypt size after refeeding, although there was an increase in villous size.

The changes in cell cycle time were accompanied by modifications in migration rate and transit time (Table 1). The migration rate was decreased after starvation, which agrees with the measurements of Hopper *et al.* (1972) and of Weibecke *et al.* (1969) made by observing the migration of villus cells labelled with ^{3}HTdr.

We have already alluded to the difficulty in interpreting mitotic accumulation results in a dynamic situation where the mitotic index is itself increasing. In the absence of changes in growth fraction and mitotic duration, any acceleration of mitotic accumulation during the stathmokinetic period must be due to a reduction in T_c. An appreciation of this point is possible from inspection of Fig. 12, which shows the data from Fig. 10*b* fitted by a quadratic expression; the fit is obviously good, and may be preferable to a linear fit, though no better than would be expected with an extra parameter. A tangent drawn to the curve at any point is equal to the rate of entry into mitosis at that point. For comparative purposes we have used a linear model in Fig. 10*a*, and the slope of the regression line in the refed animals is significantly greater than that in the starved animals. Because of the shorter time factor, stathmokinetic experiments are preferable to longer term labelling experiments in situations where cell cycle parameters are changing rapidly. Against this must be balanced the less complete form of the data, and the possibility of changes in the rate of entry into mitosis even over so short a period as 2·5 hours.

In conclusion, we have found that cycle parameter changes are the chief means whereby the hypoproliferative response to starvation is produced in the rat small intestinal mucosa, and changes in T_c maybe more important in crypt kinetic reactions

than previously realized (Wright *et al.* 1972*a*), particularly in hypoproductive states. Furthermore Lesher & Bauman (1969) have reported a reduction in T_c from 12 to 7·5 hours in the small bowel of irradiated rats, and Wright, Watson, Morley, Appleton, Marks & Douglas (1973) have recently demonstrated a reduction in T_c in patients with coeliac disease. Cell cycle time changes may also emerge as important factors in increases of cell production.

SUMMARY

Male rats were starved for a period of 96 hours. Measurements of crypt cell population showed a small reduction during starvation. The growth fraction remained constant, but the total number of proliferating cells per crypt fell as a consequence of the reduction in crypt population. Both labelling and mitotic indices fell throughout the starvation period. The cell cycle time (T_c), measured by a stathmokinetic technique using vincristine, was increased from 10·4 hours in control rats to 14·7 hours after 96 hours' starvation.

Upon refeeding, the proliferative indices were observed to rise. After a small initial fluctuation, the growth fraction remained constant. The crypt population remained substantially unchanged. Sixteen hours after refeeding, the cell cycle time was reduced to 6·5 hours.

The hypoproliferative response to starvation is mediated solely by an increase in cell cycle time, and the response to refeeding is interpreted in terms of a reduction in T_c. Changes in the size of the proliferating population are considered not to play an important role in either response, although it is not possible to exclude entirely the presence of resting cells in the proliferative compartment itself.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We are grateful to Miss Elizabeth Robinion, A.I.M.L.T. for skilled technical assistance.

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David Appleton

THE EFFECT OF A SINGLE INJECTION OF HYDROXYUREA ON CELL POPULATION KINETICS IN THE SMALL BOWEL MUCOSA OF THE RAT

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(*Received* 17 *May* 1976; *revision received* 30 *June* 1976)

ABSTRACT

The effect of a single injection of hydroxyurea (HU) on cell population kinetics in the jejunal crypt of the rat was studied using autoradiography with tritiated thymidine and metaphase arrest with vincristine. HU appeared to act selectively on cells in the S phase producing inhibition of DNA synthesis and cell death. The deficit in proliferating cells was made good by a decrease in cell cycle time and an increase in growth fraction. Particular attention was paid to the basal, slowly cycling (and possibly clonogenic) crypt cells; early in the recovery sequence an increase in cell production rate was found in the base of the crypt.

It is proposed that basal crypt cells, having survived cycle-specific insult because of long cell cycle times, proceed to repopulate the depleted proliferative compartment.

INTRODUCTION

The cells of the small bowel mucosa are notably sensitive to irradiation and cytotoxic chemotherapy, but Lamerton (1972) suggested that small intestinal epithelium is less readily damaged than would be expected from examination of its cell kinetics; in the crypts of Lieberkühn of the rodent the cell cycle time is short and the growth fraction is high, a situation which, relatively speaking, also occurs in man (Wright *et al.*, 1973). Lamerton (1972) proposed that a subpopulation of crypt cells are possessed of comparatively long cell cycle times, are less likely to be in a sensitive phase when exposed to irradiation or chemotherapy, and are thus available for crypt repopulation.

Experimental results indicate that these cells are situated in the base of the crypt (Cairnie, Lamerton & Steel, 1965; Wright, Morley & Appleton, 1972; Al-Dewachi *et al.*, 1974), and it

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is of interest that Hendry & Potten (1974) have localized the clonogenic crypt cells to the basal portion of the mouse crypt. Furthermore, under continuous labelling with tritiated thymidine (^{3}H-TdR), Potten, Kovacs & Hamilton (1974) demonstrated that basal crypt cells of the mouse show later labelling, indicating a relatively prolonged G_1 phase in these cells. A reasonable hypothesis is that the basal clonogenic cells are longer cycling, and consequently will survive phase-specific damage to repopulate the crypt.

This proposal has been investigated by a study of the effect of cytotoxic chemotherapy on cell population kinetics in the basal, and other portions of the jejunal crypt of the rat: detailed information is available for comparison in the normal animal (Wright *et al.*, 1972, 1975; Al-Dewachi *et al.*, 1974). The agent chosen was hydroxyurea (HU), given as a single dose. Hydroxyurea stops DNA synthesis by acting on nucleotide reductase (Moore, 1968) thus inhibiting the reduction of ribonucleotide diphosphates to deoxyribonucleotide diphosphates (Frenkel & Arthur, 1967). Farber & Baserga (1969) have demonstrated its cytotoxic effect on rapidly renewing tissues, while Dethlefsen & Riley (1973) have reported its action on the whole crypt of the mouse.

MATERIALS AND METHODS

Male albino Wistar rats weighing 150–200 g were used throughout. Animals received 1840 mg/kg body weight of HU (Sigma, U.S.A.) by intraperitoneal injection. In the labelling experiments rats received 0·5 μCi/g body weight of tritiated thymidine (Radiochemical Centre, Amersham, England), and in the stathmokinetic procedures 1 mg/kg body weight of vincristine sulphate (Oncovin, Lilly) was given, also by intraperitoneal injection. The animals were killed by cervical dislocation, and samples of small bowel were removed at a site immediately distal to the ligament of Treitz. Specimens were fixed in Carnoy's fluid for 6 hr. Paraffin sections were cut at a thickness of 3 μm and, where appropriate, autoradiographs were prepared as previously described (Al-Dewachi *et al.*, 1974). Sections were routinely stained by Harris's haematoxylin.

Experiment 1

Forty rats were each given a single injection of HU, and an animal was killed every hour during the first 24 hr, then every 3 hr up to 72 hr after injection. Animals received ^{3}H-TdR 1 hr before death. In each animal 100 axially sectioned crypts were selected for analysis of the distribution of labelled and mitotic cells, as previously described (Wright *et al.*, 1972, 1975). Labelling index distribution curves were constructed in each animal, to follow the proliferative response at each cell position in the crypt. Histological changes were studied in sections stained with haematoxylin and eosin. Sections cut tangentially to the bowel lumen were prepared for the determination of the column count (Wright *et al.*, 1975), the number of cells in the circumference of the crypt.

Experiment 2

A fraction of labelled mitoses (FLM) experiment was carried out at 15 hr after HU administration: at this time animals were given ^{3}H-TdR, and an animal was killed every 30 min for the first 12 hr, and subsequently every hour up to 28 hr after ^{3}H-TdR administration. Axially sectioned crypts were selected, and the left-hand cell column was divided into six vertical segments comprising sequentially cell positions 1–4, 5–8, 9–12, 13–16, 17–20 and positions

21 and above. Counts of labelled and unlabelled metaphases and anaphases were made as previously described (Al-Dewachi *et al.*, 1974), and the resulting FLM curves were analysed by the Gilbert (1972) program.

Experiment 3

Stathmokinetic experiments were carried out at 15 hr and 20 hr respectively after HU administration. Animals were given 1 mg/kg body weight of vincristine sulphate, and killed serially, one every 15 min until 2·5 hr after vincristine. In each animal the positions in the crypt column of mitotic nuclei were quantified in 100 axially sectioned crypts, and the data were analysed as previously described (Wright *et al.*, 1972).

RESULTS

Histological observations

Degenerative changes were confined to the cells of the crypt of Lieberkühn, and here the changes were localized to the proliferative zone; the cells occupying the upper third of the crypt, as well as those in the bottom few cell positions, did not appear to be affected. Villous epithelium showed no signs of damage and remained indistinguishable from the normal. Following administration of HU manifest damage to crypt cells was first seen at 2 hr and reached maximum severity at 7 hr. Thereafter the proportion of necrotic cells showed a steady decrease, and by 24 hr few remained. The affected cells showed pyknosis and karyorrhexis with condensed, fragmented, basophilic nuclear material surrounded by deeply eosinophilic cytoplasm or by an apparently empty space.

None of the necrotic cells or fragmented nuclei appeared to take up ^{3}H-TdR.

Morphometric parameters

At 10 hr after HU the number of cells per crypt column had decreased from a control value of 32·9 ± 0·2 (SE) to 28·6 ± 0·2 cells, and there was a similar reduction in the circumference of the crypt, as indicated by changes in the column count (Fig. 1). After 10 hr there was an increase in both values, the column count reaching control levels by about 30 hr after HU administration; the crypt column length rose more slowly and was only nearing control values by the end of the experiment (72 hr).

Labelling and mitotic indices

Hydroxyurea, in the dose used, caused an immediate inhibition of DNA synthesis, with total abolition of labelling within 1 hr of HU administration (Fig. 2), while the mitotic index, though reduced at 1 hr, fell to zero only at 3 hr; this difference presumably reflects the time taken for cells, already in G_2 or mitosis at the time of HU injection, to complete the mitotic process.

Starting at 10 hr, the labelling index rose rapidly to reach a peak of about 40%, slightly above control values, at 20 hr. This was followed by a fall to 30% at 30 hr, a level which was maintained until the end of the experiment. Mitotic activity also resumed at about 10 hr, and the mitotic index rose to a peak of 8% and then fell to control levels at about 32 hr.

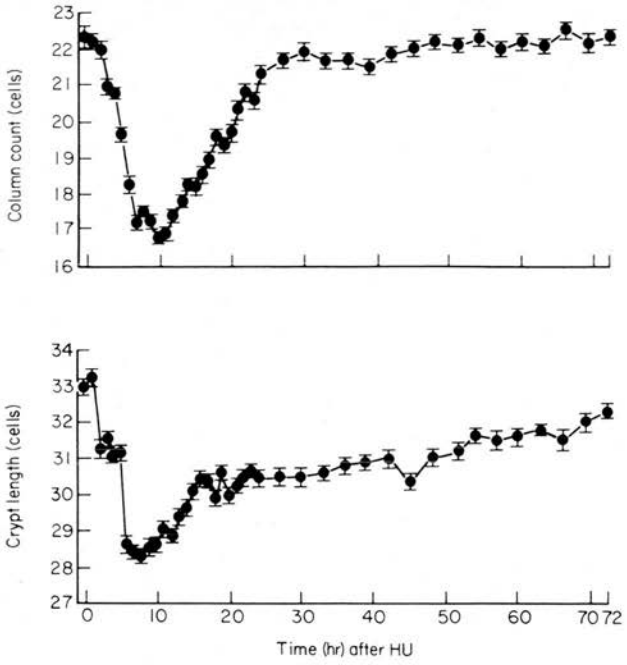

FIG. 1. The changes in crypt column length and column count in the rat jejunal crypt after a single dose of hydroxyurea. The values shown are mean values with SE. Each reading indicates one animal.

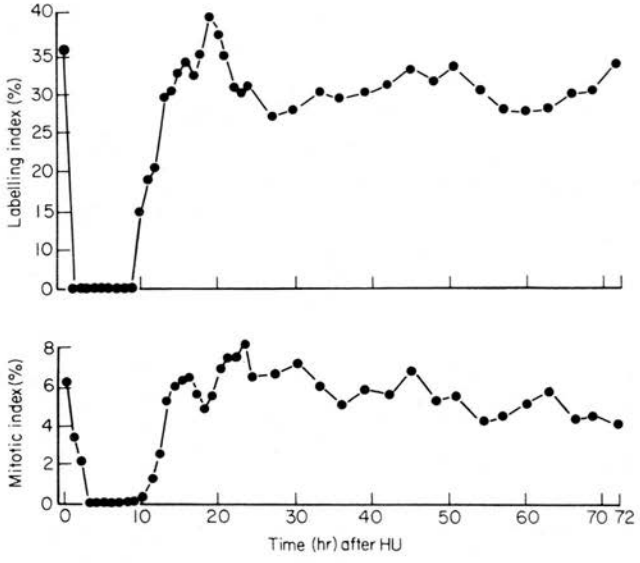

FIG. 2. Labelling and mitotic indices in the rat jejunal crypt after a single injection of hydroxyurea.

Distribution of labelled cells in the crypt

The distributions of labelled cells in the standardized crypt are shown for the period 10–24 hr after HU in Fig. 3. When crypt analysis became possible at 10 hr, low labelling indices (I_s) were found at each cell position; at position 1 I_s was below 5%, and in the proliferative compartment peak values were reduced to around 35% (normal value *c.* 60%; Wright *et al.*, 1975).

In the next 2 hr there were small increases in I_s at most cell positions, but indices above cell position 20 remained at zero, suggesting that no increase in growth fraction had occurred, by upward movement of the cut-off position.

At 13 hr there was a large increase in the labelling index in lower cell positions, particularly positions 1–8, with a peak value of 78% at cell position 5; cell positions 9 and above showed the low indices apparent at 11 and 12 hr after HU. At 14, 15 and 16 hr after HU the increased labelling indices in the lower cell positions were largely maintained, but I_s in cell positions 9 and above gradually increased to match the lower cell positions, and a smooth

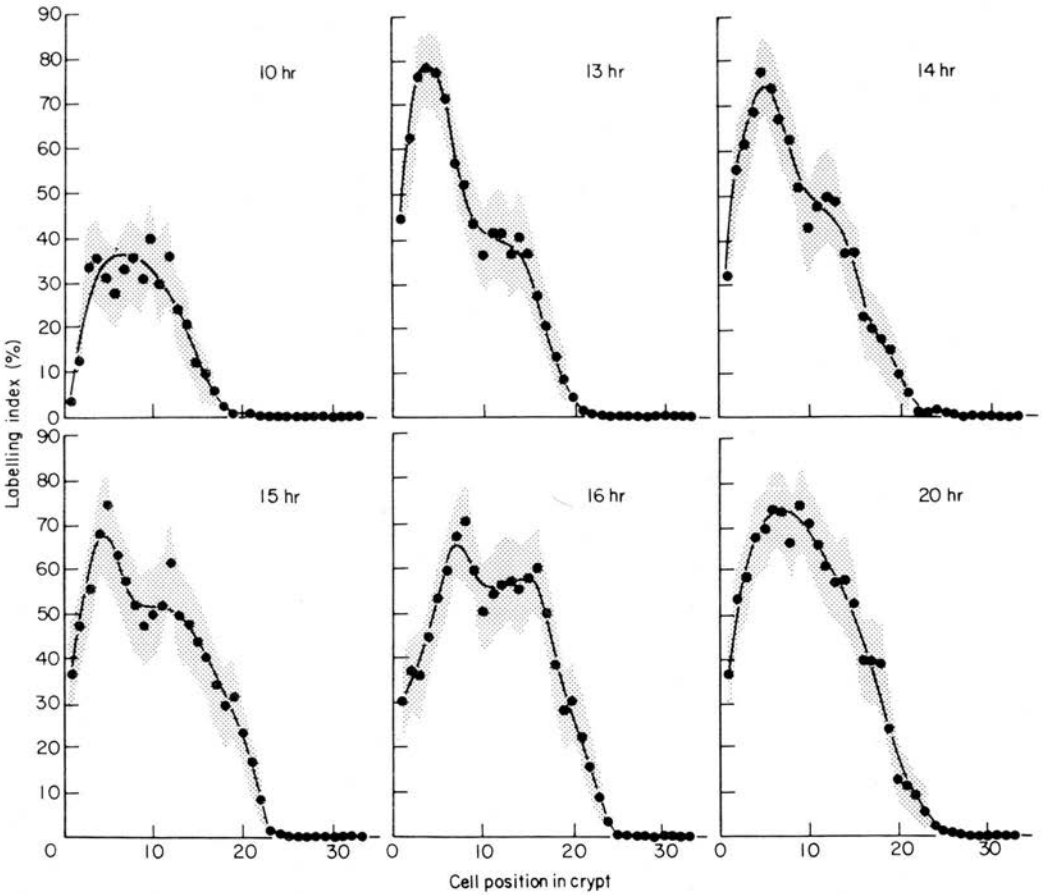

FIG. 3. Labelling index distribution curves in jejunal crypts of rats at the times indicated after a single injection of hydroxyurea. The shaded areas indicate 95% confidence limits for the points.

labelling index distribution curve was again realized by 19 hr after HU. This increase in I_s in higher cell positions indicates an upward movement of the cut-off position, and an increase in the size of the proliferative compartment at the expense of the maturation compartment; in effect an increase in growth fraction occurs at this point.

The labelling index reached a maximum at about 20 hr, and this is associated with high I_s values in the proliferative compartment, which exceed 80%; the subsequent fall in labelling index is accompanied by a decrease in peak I_s values within the proliferative compartment.

FLM data

The FLM curves obtained by injecting ^{3}H-TdR at 15 hr after hydroxyurea are shown in Fig. 4 for the designated segments of the crypt. The mean durations of cycle parameters, with standard errors and coefficients of variation, are given in Table 1. The mean cell cycle time for the whole crypt is 9·6 hr compared with the control value of 11·3 hr (Al-Dewachi *et al.*, 1974). Using the same criteria as before (Al-Dewachi *et al.*, 1974), the difference is considered highly significant; this decrease appears to be brought about largely by a decrease in t_S.

In all cases the Gilbert program appears to fit the first wave of labelled mitoses reasonably well, and in some curves the second wave is also well depicted. However, in cell positions 5–8, 9–12 and 17–20, the line fitted by the computer is below the later experimental points. A

TABLE 1. Mean values (±SE) of the durations of the cell cycle in different portions of the jejunal crypts of male Wistar rats 15 hr after hydroxyurea administration

	T_C	T_{G1}	t_S	t_{G2}	CV_C	CV_{G2}	CV_{S+G2}
Cell positions 1–4							
Mean	10·9	2·9	6·6	1·3	24·8	22	19
SE	0·1	0·1	0·1	0·02	1·1	4	1
Cell positions 5–8							
Mean	10·9	4·0	5·6	1·3	24	50	25
SE	0·2	0·2	0·1	0·05	2	7	2
Cell positions 9–12							
Mean	9·2	2·8	5·1	1·4	25	55	20
SE	0·2	0·2	0·2	0·1	3	9	3
Cell positions 13–16							
Mean	9·2	2·9	5·0	1·2	21	24	25
SE	0·2	0·2	0·1	0·04	2	7	3
Cell positions 17–20							
Mean	9·2	2·6	5·2	1·3	21	37	30
SE	0·2	0·2	0·2	0·1	2	9	4
Cell positions above 20							
Mean	8·9	2·3	5·7	1·3	21	47	29
SE	0·2	0·1	0·2	0·05	2	7	3
Whole crypt							
Mean	9·6	2·9	5·4	1·3	24	40	26
SE	0·2	0·1	0·1	0·03	2	5	2

* CV represents coefficient of variation and is expressed in %.

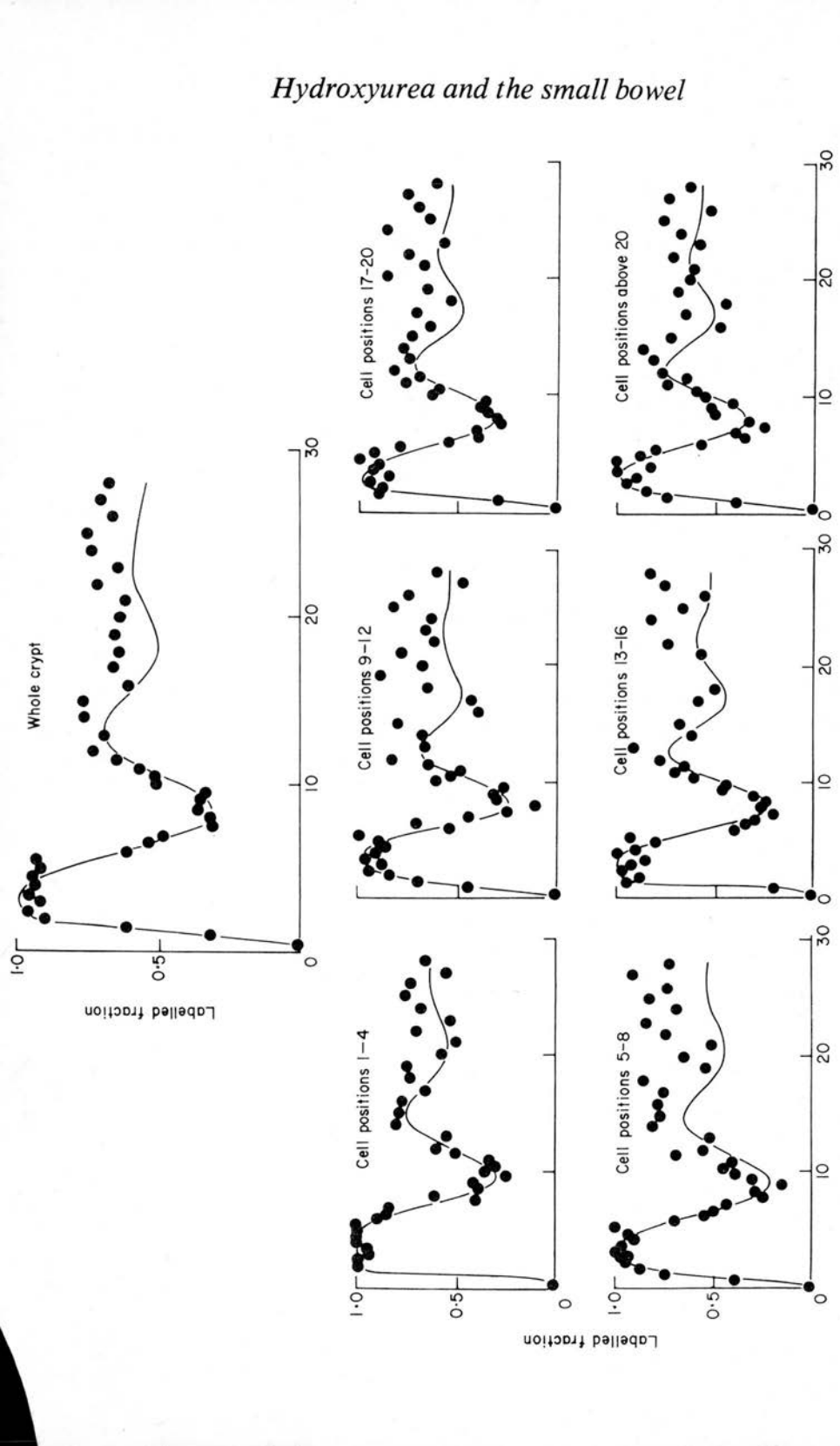

FIG. 4. Fraction of labelled mitoses curves of the cell positions indicated in the rat jejunal crypt. Tritiated thymidine was injected at 15 hr after hydroxyurea. The solid points are the experimental values, while the lines are fitted by the Gilbert program.

considerable change is seen in the cell cycle time in cell positions 1–4, with a decrease from 15·4 hr to 10.9 hr. This difference in T_C, which is highly significant, is mostly at the expense of t_{G1}, which is nearly halved.

In the higher cell positions there are smaller but significant decreases in T_C compared with controls, caused by changes in t_S and t_{G1}; t_{G2} remains close to control values. In the HU treated animals, T_C in cell positions 1–4 ad 5–8 is longer compared with values obtained for higher cell positions.

Stathmokinetic results

The mitotic index accumulation lines, fitted assuming an exponential age distribution, at 15 hr and 20 hr after HU administration are shown in Fig. 5. Despite the fact that in the absence of vincristine administration the mitotic index is increasing over this period (Fig. 2), after vincristine the mitotic index appeared to rise linearly. The crude rate of entry into mitosis was 10 and 11% of cells per hour, compared with a control value of 11% per hour. It should be remembered that these values are probably overestimated, since the mitotic index is itself increasing over the experimental period.

Mitotic accumulation graphs were constructed for each cell position, and the rate of entry into mitosis (% cells/hr) was measured; these values are shown in Fig. 6. At 15 hr after HU there is an increase in the birth rate in lower cell positions, particularly positions 3 and 4. Smaller values are then found in the proliferative compartment, but there is a further peak between cell positions 13 and 17. The similarity between this curve and the labelling index distribution curves at 15 hr and 16 hr after HU (Fig. 3) should be noted. At 20 hr birth rates are high throughout the proliferative compartment, again reflecting the labelling index distribution curve.

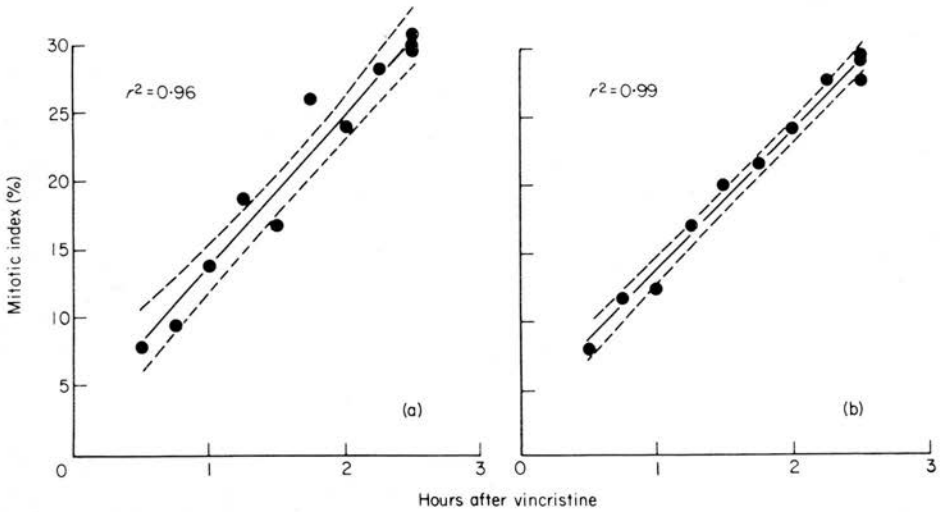

FIG. 5. Mitotic accumulation lines with vincristine (a) at 20 hr and (b) at 15 hr after hydroxyurea. The dashed lines indicate 95% confidence limits for the line.

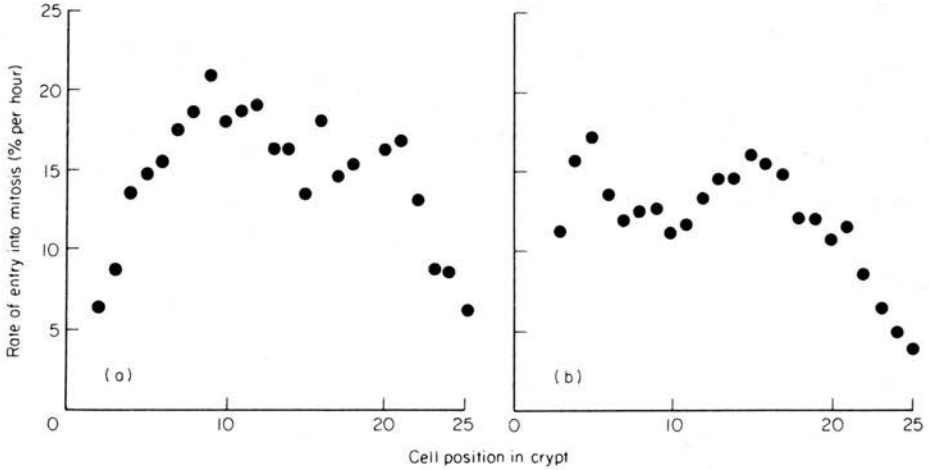

FIG. 6. The rate of entry into mitosis plotted as a function of cell position in the crypt at 20 hr (a) and 15 hr (b) after a single injection of hydroxyurea.

DISCUSSION

Mechanism of action of hydroxyurea

It is apparent from measurements of the crypt column length and column count that, in the doses used, hydroxyurea produces cell loss in the proliferative portion of the crypt. Inspection of the responses of the labelling and mitotic indices (Fig. 2) shows that total inhibition of proliferative activity lasted for about 9 hr after injection; the immediate fall in the labelling index suggests a direct action on cells synthesizing DNA; the later decrease in the mitotic index, with values reaching zero 2–3 hr after HU administration, also supports this interpretation. There is little evidence of a G_1–S block at this dosage: if cells were only blocked at the G_1–S boundary and did not stop or die in S, then the mitotic index would fall only after a time approximating to $t_S + t_{G2}$ (or about 8 hr). Some authors (Hagemann & Lesher, 1971) have suggested that HU, in addition to its inhibiting and cytotoxic effect on DNA synthesizing cells, induces a G_1–S block which is partially responsible for the fall in ^{3}H-TdR incorporation, and for the synchronization which follows. In the present study there is little direct evidence of any large G_1–S block; the peak labelling and mitotic indices attained during recovery are little higher than the control values, indicating a very small degree of synchrony. In addition, the increases in I_s and I_m during recovery are probably too close together to be due solely to cells, previously blocked in G_1, passing through S, G_2 and into mitosis. Plager (1975) considered that one reason for the lack of an apparent G_1–S block with higher doses of HU was that, if cells are blocked at the G_1–S boundary or in early S, they are also killed. In this context, Bachetti & Whitmore (1969) showed that mouse L cells were more sensitive to HU in the early part of S than in the later part of the S phase.

If release of a G_1–S block is not the major factor concerned in the recovery of the I_s and I_m, what is the mechanism? It can be seen that mitoses appear very soon after the resumption of DNA synthesis. This is consistent with cells being blocked at various points in S (possibly late), and resuming DNA synthesis and movement around the cycle at about 9 hr after HU.

But we consider that most cells blocked in DNA synthesis are killed, since there is insufficient indication of synchrony in the proliferative indices to accord with large numbers of cells suddenly resuming DNA synthesis. Thus it appears that the early part of the recovery of the labelling index is due to the resumption of DNA synthesis in surviving cells, with G_1 cells entering DNA synthesis also. However, it must be conceded that these conclusions are based on indirect evidence. Confirmatory studies are in progress which are quantifying pyknotic cells in microdissected crypts; this will be correlated with the number of cells which have entered S during the cytotoxic period. This study will then differentiate between those cells killed by HU, and those which, although in S at the relevant time, were prevented from synthesizing DNA (Fig. 2), but survived.

Proliferative responses in the crypt

In a previous study of the variation in cell cycle time with cell position in the crypt, Al-Dewachi *et al.* (1974), using the FLM technique, showed extended cell cycle times at basal crypt cells, but throughout the remainder of the proliferative compartment the cell cycle time was about 11 hr.

The FLM data indicate a reduction in the cell cycle time during recovery from HU. Within the crypt, the most prominent decrease in T_c was seen in the lowest cell positions, i.e. within the Paneth cell zone, where the putative clonogenic crypt cells are found (Hendry & Potten, 1974). For the whole crypt the CV_c was 24%, compared with a control value of 21%, indicating a similar distribution of cell cycle times. However, in basal cell positions (1–4), the CV_c is reduced from 30·5% to 24·8%, suggesting a tighter distribution of individual cell cycle times in this portion of the crypt. As noted above, the computer fitted line diverges from the experimental points after the first peak; the program does give most weight to the early readings, which may account for some of the discrepancy.

More information on the behaviour of the basal cell positions during recovery is obtained from the labelling index distribution curve. At 12 hr after HU, I_s in lower cell positions is increasing, but 1 hr later there is practically a doubling of I_s at most cell positions between 1 and 6. From 14 to 16 hr the high I_s values in lower cell positions are maintained, but there is a gradual increase in I_s in higher positions so that after 19 hr a smooth curve again prevails. It would appear that the increase in I_s in basal positions is due to an increased cell production rate, and not to any change in the duration of S, since t_s as measured from the FLM curve is decreased rather than increased.

We suggest that the first active responses to death of proliferative cells is an increase in cell production rate in the lower cell positions; this is probably produced by a decrease in the cell cycle time, since the FLM experiment, started at 15 hr after HU, showed a decrease in T_c in this part of the crypt. It would be difficult to exclude any contribution to this increase made by non-cycling cells coming into cycle in this portion of the crypt, although calculations from data on control animals (Al-Dewachi *et al.*, 1974; Wright *et al.*, 1975) indicate that few non-cycling cells are likely to be found here. The increase in cell production rate would augment the efflux into the proliferative compartment, replacing the cells destroyed. These repopulating cells also have a shorter cell cycle time, as indicated by the FLM curve and the high I_s values in the proliferative compartment at 19–20 hr.

The increase in I_s in higher cell positions produces an upward movement of the cut-off position, and thus an increase in the proportion of proliferating cells per crypt occurs, contributing to the increase in labelling index in recovery.

The peaks in proliferative activity occurred between 20 and 24 hr after HU, whereas the crypt length was only approaching control levels towards the end of the experiment. It is interesting to note that the column count regains its previous value before the crypt column length. Since the column count was always obtained from levels in the crypt where mitoses were seen, this may mean that during recovery mitotic planes are so disposed to give increases in girth before length.

The present data allow the suggestion that, after death of proliferating crypt cells, the basal crypt cells assist in the repopulation of the depleted proliferative compartment. At the dosage used, inhibition of DNA synthesis lasted for 9 hr, so that only those cells entering DNA synthesis in this time were at risk of being killed. A next step will be to follow crypt repopulation when all cells in the proliferative compartment have been put at risk, using a specially designed dosage schedule.

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Virchows Arch. B Cell Path. 28, 339–350 (1978)

Virchows Archiv B
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The Effect of Single and of Multiple Doses of Prednisolone Tertiary Butyl Acetate on Cell Population Kinetics in the Small Bowel Mucosa of the Rat

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Summary. The effect of single and of multiple doses of prednisolone upon cell population kinetics in the rat jejunal crypt was investigated, using autoradiography and stathmokinetic techniques with vincristine. Single injections of prednisolone (2.5 mg/kg body weight) induced a depression in both flash thymidine labelling and mitotic indices; this change was shown to be due to a decreased cell production rate. Recovery of these proliferative indices occurred over seven days after injection; measurement of crypt size parameters showed a transient decrease in crypt population.

Multiple daily injections of prednisolone (1 mg/kg body weight) produced a more sustained decrease in labelling and mitotic indices, which lasted as long as injections were continued (7 days); stathmokinetic techniques showed decreases in cell production rates, and the crypt population was also depressed throughout this period.

It is concluded that prednisolone depresses cell proliferative rates in rat jejunal mucosa.

Key words: Intestine – Prednisolone – Kinetics – Thymidine – Vincristine.

Introduction

Adrenocortical hormones, notably the glucocorticoids, have previously been shown to inhibit mitotic activity in several tissues, e.g. mouse epidermis (Bullough, 1965), mouse squamous forestomach (Frankfurt, 1968), and adrenal cortex (Wright, Appleton and Morley, 1974). Sequential studies of the action of such steroids on mitotic index and tritiated thymidine (^{3}HTdR) flash labelling index demonstrate that glucocorticoids inhibit the flow of G_1 cells into DNA synthesis (Frankfurt, 1968; Wright et al., 1974).

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0340-6075/78/0028/0339/$02.40

In keeping with these findings is the observation that prednisolone-21-phosphate, a synthetic hormone with both glucocorticoid and mineralocorticoid activity, can cause small intestinal crypt hypoplasia (Wall and Peters, 1971). Tutton (1973) reported that a single injection of prednisolone tertiary butyl acetate decreases the cell cycle time in rat small bowel, from 17 h in control to 8 or 9 h in treated animals, on the 7th day after administration. It thus becomes important to ascertain whether prednisolone tertiary butyl acetate really differs from other steroids in stimulating rather than inhibiting cell proliferation. In this article we report the results of an autoradiographic and stathmokinetic study of the action of a single dose and of repeated daily doses of prednisolone on the rat jejunal mucosa. We find that prednisolone depresses cell proliferation in small intestinal crypts by decreasing the crypt cell production rate; recovery from a single injection occurs over 7 days, but with multiple daily injections proliferative rates remained depressed.

Materials and Methods

Male albino Wistar rats aged 3 months and weighing 200–400 g were used throughout; they were maintained on Spiller's rat cubes and water ad libitum. All injections were given intraperitoneally. In the labelling experiments the dose of ^{3}HTdR was 0.5 μCi per gram body weight. The rats were injected with prednisolone tertiary butyl acetate (Codelcortone-TBA, Merck, Sharp and Dohme Ltd.) by the intraperitoneal route. They were killed by cervical disolocation and samples of upper jejunum were removed from a site immediately distal to the ligament of Treitz, to be fixed in Carnoy's fluid for 6 h. The tissue was embedded in paraffin wax and sections were cut at a thickness of 3 μm: autoradiographs were prepared using Kodak A.R. 10 stripping films as previously described (Al-Dewachi et al., 1974) employing an exposure period of 28 days.

Experiment 1

Twenty-two rats received a single injection of prednisolone in a dosage of 2.5 mg/kg body weight at 0900h. One animal was killed every 8 h over a period of 7 days after the injection of prednisolone, each having received an injection of ^{3}HTdR one hour before death.

Experiment 2

Stathmokinetic studies were carried out at 32 h, 96 h and 7 days after the single injection of prednisolone: at each time 11 rats were injected with vincristine sulphate (Oncovin, Eli Lilly) in a dosage of 1 mg/kg body weight, and were killed serially over 2.5 h after injection of vincristine.

Experiment 3

Twenty-two rats were given daily injections of prednisolone in a dosage of 1 mg/kg body weight at 0900h. One animal was killed every 8 h over the seven days after commencing injections, each having received an injection of ^{3}HTdR 1 h before death.

Experiment 4

Three groups of 11 rats were used for stathmokinetic studies at 32 h, 96 h and 7 days after commencing daily injections of prednisolone, using the same experimental protocol as in experiment two.

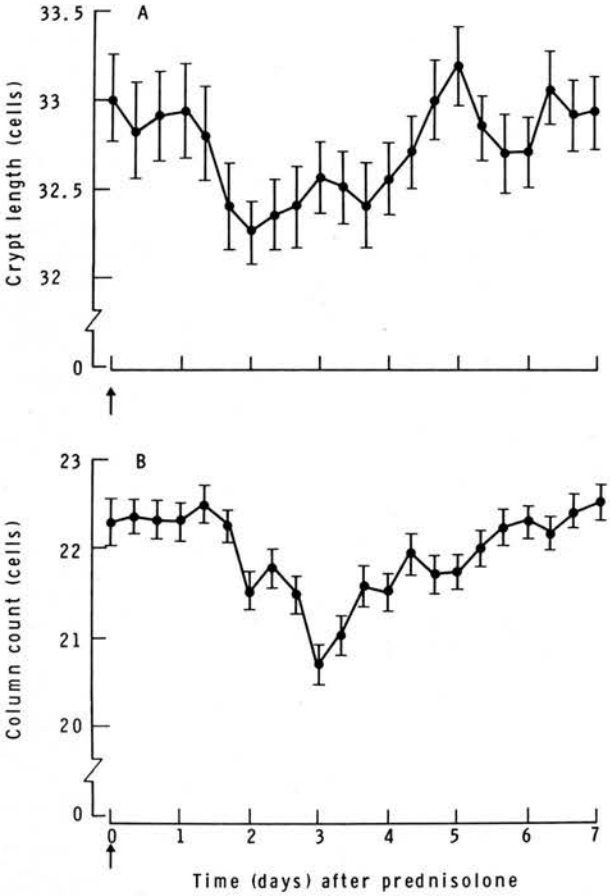


Fig. 1 A and B. The effects of a single injection of prednisolone (2.5 mg/kg body weight) on the crypt column length (±S.E.); **A** and the column count (±S.E.); **B** in rat jejunum. S.E. are for individual animals

In both labelling and stathmokinetic experiments, 100 axially sectioned crypts were selected, i.e. those in which the base, middle and apex of the crypt were all in the plane of the section. The cells in the left hand *crypt column* were numbered from the base upwards to the crypt-villus junction and the cell positions of labelled and mitotic cells were recorded. The data were analysed by a modification (Wright et al., 1972a, b) of a computer program designed by Cairnie and Bentley (1967), which compensates for differences in the distribution of labelled and mitotic cells in crypts of different lengths. The blocks were then turned through 90° and sections were cut tangentially to the bowel lumen so that the *column count*, or number of cells in crypt cross-sections, could be measured in 100 circular crypt cross-sections per animal (Wright et al., 1975).

In the labelling index distribution curve, the cell position at which the labelling index falls to half the peak value attained is useful as a reference point on the curve, and if the number of cell positions below this point is divided by the total number of cells in the standardised crypt column a useful estimate of the cut-off position is obtained (Cairnie et al., 1965), which describes the size of the proliferative compartment.

In stathmokinetic experiments, because of uncertainties concerning the onset of metaphase arrest during the early period of vincristine action (Al-Dewachi et al., 1975), the readings were

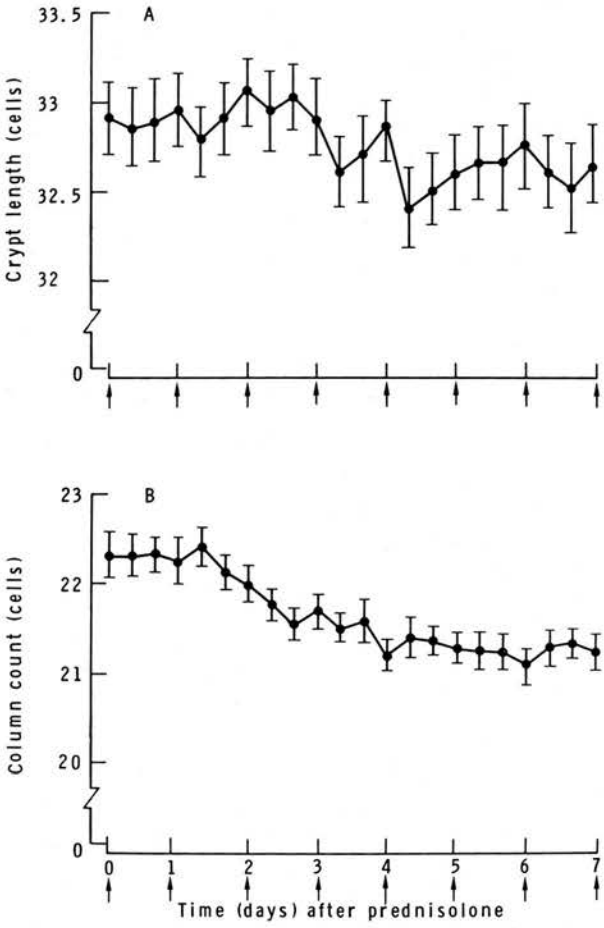


Fig. 2 A and B. The effects of multiple doses of prednisolone (1 mg/kg body weight) given daily at 0900 h (indicated by arrows beneath abscissa), on the crypt column length (±S.E.); **A** and the column count (±S.E.); **B** in rat jejunum. S.E. are for individual animals

begun at 30 min after injection. By plotting the mitotic index for each cell position in the crypt column against duration since vincristine, it is possible to calculate mitotic accumulation curves for each cell position (Wright et al., 1972b; Al-Dewachi et al., 1976). These curves were fitted assuming an exponential age distribution and the birth rates calculated from the fitted curves.

Results

Morphometric Findings

The changes in crypt column length and in crypt column count are shown for the animals treated with a single injection of prednisolone in Figure 1. The standard errors given are for the counts on individual animals. By 2 days

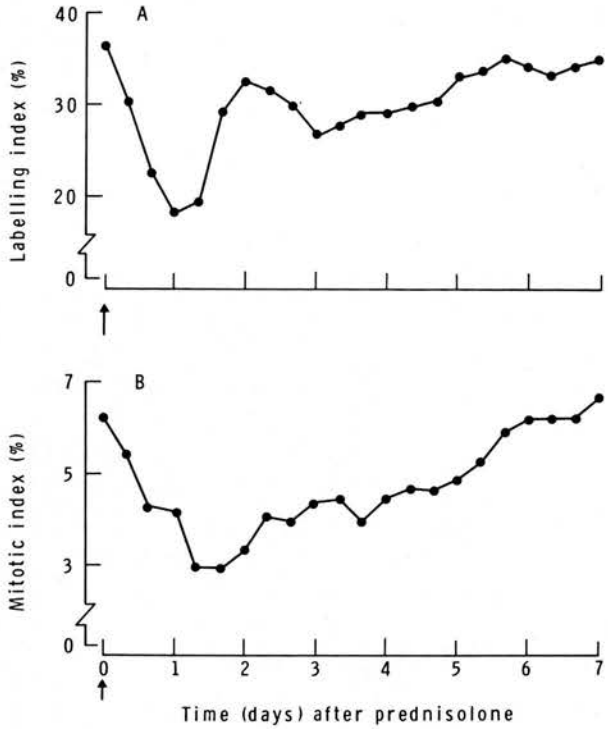


Fig. 3 A and B. The flash tritiated thymidine labelling index **A** and the mitotic index **B** in the rat jejunum after a single injection of prednisolone (2.5 mg/kg body weight). The data is for the whole crypt

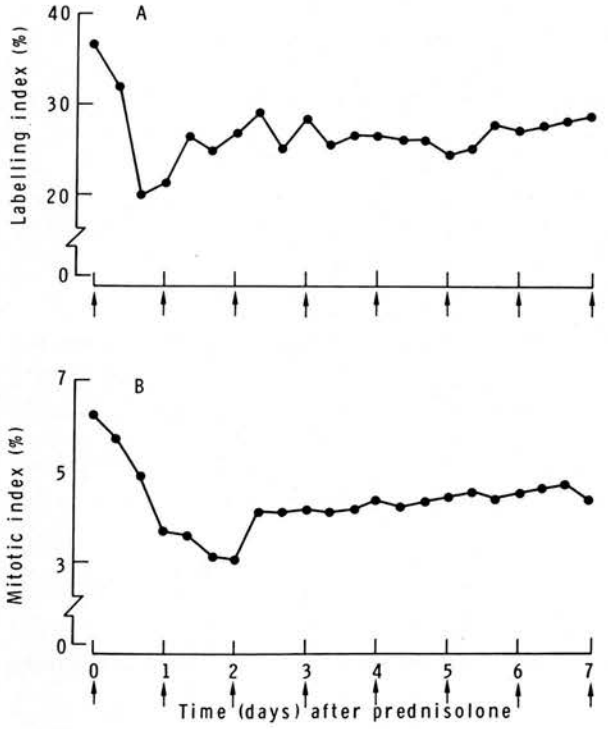


Fig. 4 A and B. The flash tritiated thymidine labelling index **A** and the mitotic index **B** in the rat jejunum after multiple daily injections of prednisolone (1 mg/kg body weight). The data is for the whole crypt

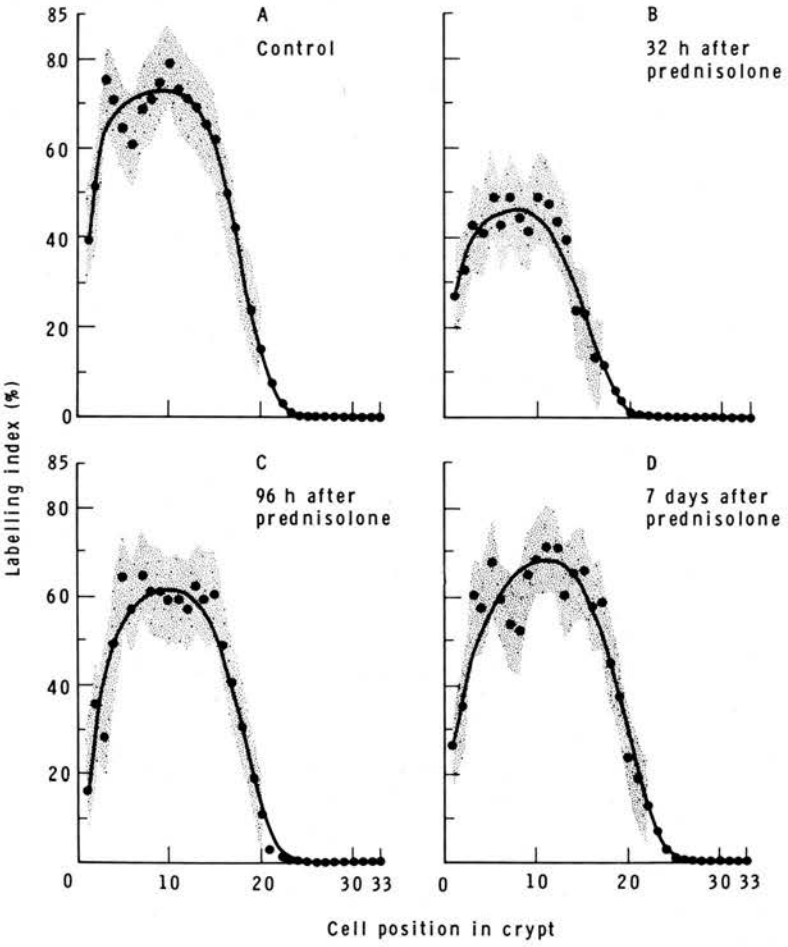


Fig. 5 A–D. A selection if labelling index distribution curves in the rat jejunum after a single injection of prednisolone (2.5 mg/kg body weight). The shaded areas indicate 95% confidence limits for the points

there is a decrease in both crypt column length and column count. These low values are then followed by a steady recovery to control levels by the seventh day. In those animals given multiple injections of prednisolone (Fig. 2), the crypt column length remains roughly constant for about 3 days, and then is rather smaller over the next 4 days; the column count remains about the same for one or two days, then decreases steadily over the remainder of the experimental period.

We may conclude that a single injection of prednisolone causes a transient decrease in the crypt population, which starts on the second day, followed by a recovery which is complete by the end of a week. Daily but smaller prednisolone injections cause a steady decline in crypt population which begins after about 2 days.

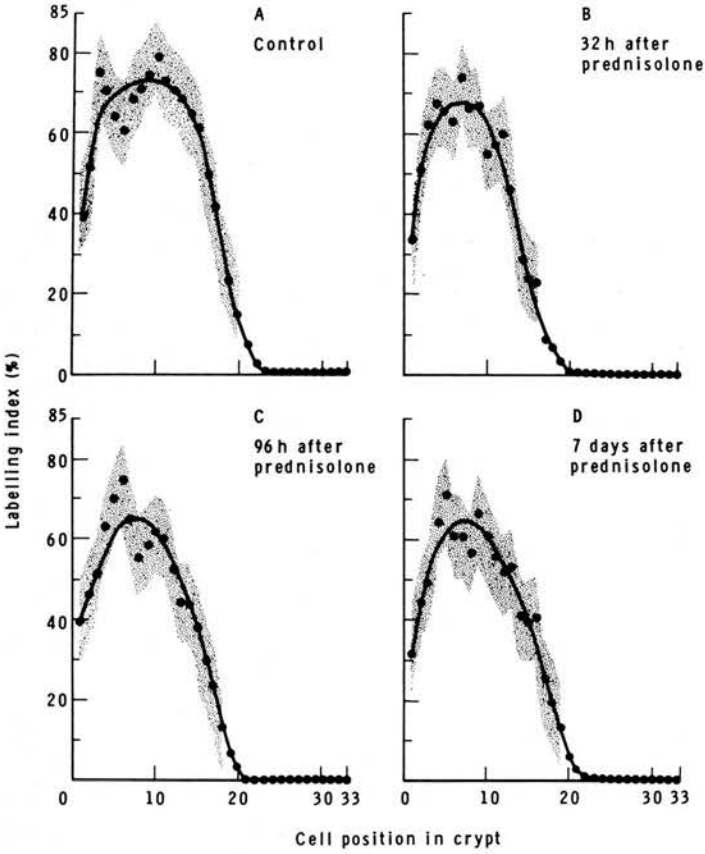
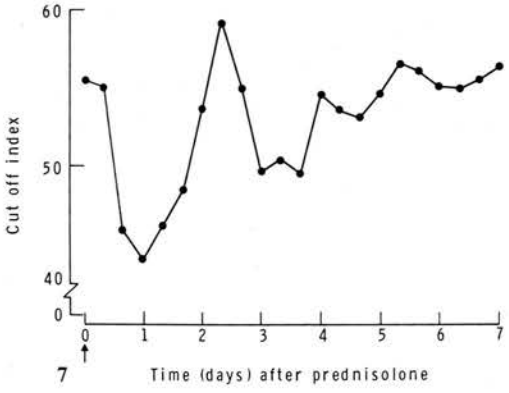


Fig. 6 A–D. Labelling index distribution curves in the rat jejunum after multiple daily injections of prednisolone (1 mg/kg body weight). The shaded areas indicate 95% confidence limits for the points

Changes in Proliferative Indices

The changes in overall labelling and mitotic indices in rats injected with a single dose of prednisolone are shown in Figure 3. Prednisolone in this dosage (2.5 mg/kg body weight) causes an immediate fall in the labelling index (I_S), from 36% in controls to 18% at 25 h after prednisolone: over the next 24 h I_S recovers sharply to around 30% and then slowly rises to reach control values after 6 days. The mitotic index (I_m) also shows an initial decrease, from 6.1% in controls to 2.9% at 40 h after prednisolone. There follows a steady increase to control values at 6 days. Figure 3 indicates that the recovery in I_S clearly precedes that of the I_m.

Figure 4 shows the changes in overall proliferative indices in the jejunal crypts of rats given multiple doses of prednisolone. The initial phases resemble the single dose results. Both indices show some recovery, but after 2 days



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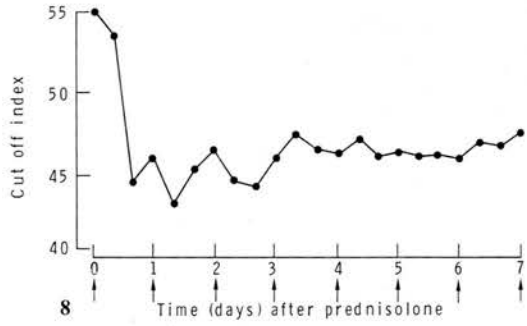

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Fig. 7. The cut-off position in the rat jejunal crypt after a single injection of prednisolone (2.5 mg/kg body weight)

Fig. 8. The cut-off position in the rat jejunal crypt after multiple daily injections of prednisolone (1 mg/kg body weight)

the labelling index becomes relatively constant at about 27%, while I_m levels off at about 4.5%.

The results indicate that a single dose of prednisolone decreases both labelling and mitotic indices in the rat jejunal crypt, which recover over 7 days. Multiple doses, however, lead to a depression in both proliferative indices, which last as long as the injections are continued (7 days).

Proliferating Crypt Populations

A selection of labelling index distribution curves in the jejunal crypts of rats injected with single and multiple doses of prednisolone is shown in Figures 5 and 6. The shaded areas indicate 95% confidence limits for the points. The changes in the cut off position, expressed as a fraction of the total crypt length, are shown in Figures 7 and 8, for the single and multiple dose regimes, respectively.

In the animals given a single injection of prednisolone, the maximum labelling index attained in the proliferative compartment drops from about 70% to 47% after 32 h (Fig. 5) at which time the index of the cut-off position has decreased from 55% to 46%; this latter decrease does not become apparent until 8 h after the injection of prednisolone (Fig. 7). After 32 h the cut-off index rises rapidly to reach a maximum of 59% at 56 h. There follows a second fall, and from 72 h onwards, there is a rise to control values.

In those animals treated with prednisolone in multiple doses (Fig. 6), the initial fall in the cut-off position resembles that in the case of the single dose. Thereafter no notable change occurs.

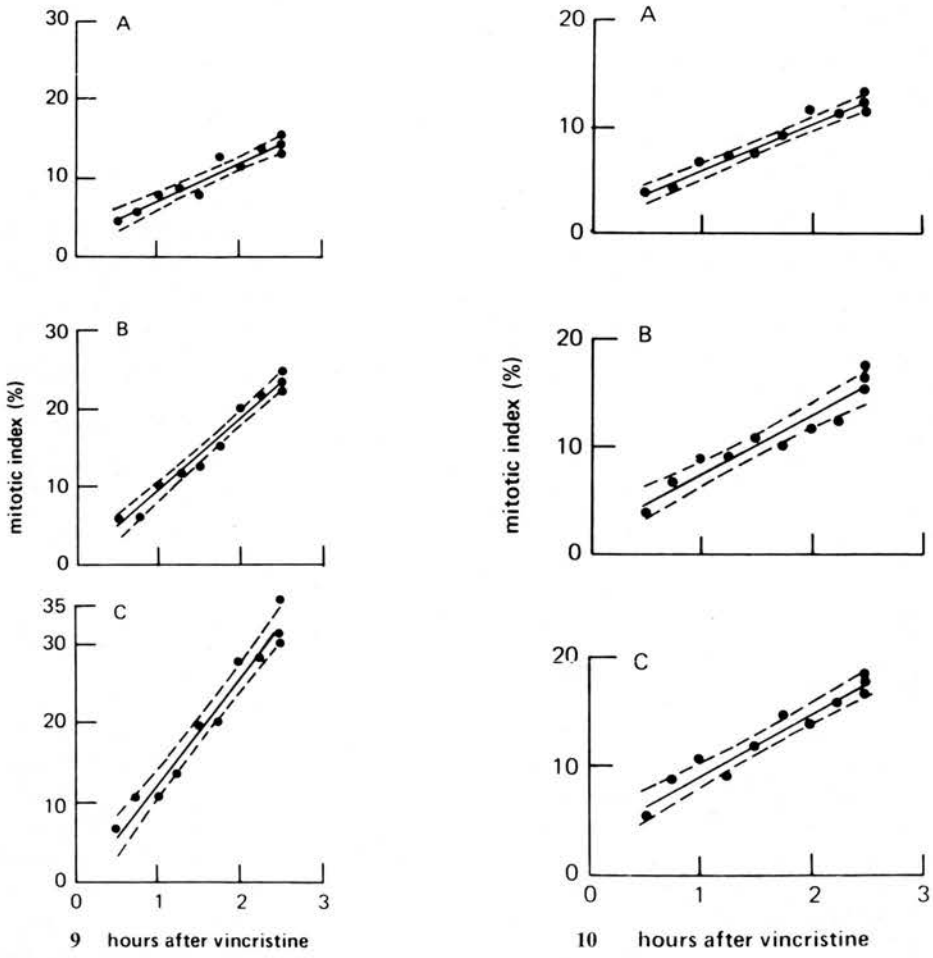

Fig. 9 A–C. Mitotic index accumulation lines following vincristine in the rat jejunum **A** 32 h, **B** 96 h and **C** 7 days after a single injection of prednisolone (2.5 mg/kg body weight). The dotted lines indicate 95% confidence limits for the lines

Fig. 10 A–C. Mitotic index accumulation lines following vincristine in the rat jejunum **A** 32 h, **B** 96 h and **C** 7 days after commencing daily injections of prednisolone (1 mg/kg body weight). The dotted lines indicate 95% confidence limits for the line

Stathmokinetic Results

Figure 9 shows the mitotic index accumulation curves after vincristine injection at 32 h, 96 h and 7 days following a single injection of prednisolone. The slopes of the lines are a reflection of the birth rate, or rate of entry into mitosis, and this equals 2.8, 5.1 and 6.9% of cells per hour respectively compared with a control value of 6.7% per h. These figures take into account the correction factor which must be applied to compensate for the fact that metaphases are

seen more often than interphase cells in the sections used (Tannock, 1967). The decrease in labelling and mitotic indices produced by a single injection of prednisolone is due to a real decrease in the rate of cell production; and during the recovery period, when the labelling and mitotic indices are rising to control levels (see Figs. 3 and 7), there is an increase in the rate of cell production.

The mitotic index accumulation curves at 32 h, 96 h and 7 days after commencing multiple prednisolone injections are shown in Figure 10. The rates of entry into mitosis at these time periods are 2.5, 3.0 and 3.1% of cells per h, compared with a control value of 6.7% per h. Multiple daily doses of prednisolone thus decrease the cell production rate of jejunal crypt cells to a level which remains essentially constant over 7 days.

Discussion

This study has shown that prednisolone induces an inhibition of cell proliferation in the rat small bowel; recovery of the crypt epithelium occurs progressively over seven days when only one injection is given, and at the end of this period, values for the cell production rate are found which reach control levels. It is clear that prednisolone decreases cell proliferation initially; however, at the end of the 7 day period, all proliferative parameters are on the increase and if this trend continued, then the results of Tutton (1973) may be explained.

Rats given daily injections of prednisolone show depressed proliferative rates throughout the course of treatment. These decreased cell production rates explain the mucosal atrophy and decrease in crypt depth induced in rat small bowel by chronic dietary administration of prednisolone (Wall and Peters, 1971). However, recent results (Batt and Peters, 1976) indicate that oral prednisolone does not affect the migration rate on jejunal villi, although migration rates on ileal villi were increased by prednisolone.

Prednisolone causes a decrease in labelling index within 8 h of injection. During the decrease in proliferative indices the readings are too far apart to allow definitive conclusions concerning the site of action of prednisolone, but during the recovery process after a single injection of prednisolone (Fig. 3), it does appear as if recovery of the labelling index has preceded that of the mitotic index by at least 8 h. These findings may be interpreted to mean that the prednisolone induces a partial block on the flow of cells from G_1 into DNA synthesis; in this case the 8 h delay between labelling and mitotic indices comprises the time required for cells, temporarily arrested in late G_1, to complete DNA synthesis, G_2 and enter mitosis after release of the block. The duration of DNA synthesis is 6.5 h, and of G_2 is 1.3 h for these rats (Al-Dewachi et al., 1974).

We have noted that prednisolone, in both single and multiple doses, reduces the birth rate. However, the birth rate reflects changes in both the growth fraction and the cell cycle time; we have, as yet, no independent measurement of the growth fraction. We can assess the relative position of the cut-off point by following the movement of the 50% peak point (Figs. 5–8), but these measurements cannot be regarded as reflecting the value of the growth fraction because the $G_1 - S$ block, which is affecting the cells in the proliferative compartment,

produces non-cycling cells in that compartment, so that in the area of the crypt where maximum labelling indices are found the growth fraction is no longer unity. However, the position of the 50% peak value can be viewed as reflecting changes in the size of the proliferative compartment brought about by upward or downward movement of the cut-off position.

During the recovery sequence from a single dose of prednisolone there is an increase in the rate of entry of cells into mitosis. This of course does not necessarily mean that cells are completing the cell cycle any faster; it may simply indicate that the partial $G_1 - S$ block is relieved, and hence more cells are flowing into mitosis, thus increasing the birth rate as measured with vincristine. However, 7 days after prednisolone injection, when the birth rate has reached control values, all blocked cells have probably re-entered the cycle. The measurements of crypt population (Fig. 1) indicate that the decreases in cell production rate are sufficient to decrease the crypt cell population.

Batt and Peters (1976) have reported that prednisolone increases the absorptive capacity and brush border enzyme activities of jejunal enterocytes. However, they could not detect changes in villus cell migration rate. Neither could they confirm the findings of Wall and Peters (1971), who showed a decrease in villus height. Batt and Peters (1976) used a dose of 0.75 mg/kg body weight, and oral administration, compared with our 1 mg/kg body weight and intraperitoneal injection. If these differences can be ignored, then the two sets of data may be incompatible, since crypt cell production rate determines villus transit time, assuming that villus cell population size remains constant, as Batt and Peters' finding of a constant villus height may suggest. Possible explanations for this disparity include changes in villus population size in the absence of changes in villus height, and possible changes in the ratio of the number of crypts to the number of villi. The present results are certainly consistent with the hypothesis of Dowling and Gleeson (1973), who believe that the adsorptive and digestive funtions of enterocytes are related to the maturity of the cells, and thus their transit time. The finding of depressed crypt cell production rates may explain increases in functional capacity by an increase in the maturity of villus cells.

These results have shown that a single dose of prednisolone produces a transitory decrease in cell production rate, and also a decrease in crypt population; the results are consistent with a partial G_1 to S flow block, and after release of this block there is a compensatory proliferative response. Multiple doses of prednisolone depressed proliferative indices and cell production rates for as long as injections were continued.

Acknowledgments. We are grateful to Mrs. E. Walker, Mrs. E. Moody and Miss D. Hardy for skilled technical assistance and to Mrs. H. Christian and Miss E.D. Davidson who typed the manuscript.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign.

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Received April 28, 1978

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Cell Proliferation in the Gastrointestinal Tract

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PITMAN MEDICAL

5.5 Some Observations on the Effect of Prolonged Asbestos Ingestion on Cell Proliferation in the Intestine of Aged Rats

R E Bolton and D R Appleton

5.5.1 INTRODUCTION

Prolonged exposure to asbestos fibre has been shown to be associated with the development of pulmonary fibrosis and the occurrence of certain forms of cancer. The links between exposure, asbestosis and lung cancer are particularly strong, with cigarette smoking having a synergistic effect on the incidence of tumours (638), and there is also good epidemiological evidence for an association between the development of mesothelioma and a history of asbestos exposure (754). Some studies have reported an increased incidence of gastrointestinal cancers in individuals occupationally exposed to asbestos (448, 639) and, although the risks appear to be less than those associated with the pulmonary and pleural tumours, there is evidence for a threefold increase in incidence among heavily exposed individuals (202). The discovery that water from Lake Superior was polluted with an asbestos-like mineral led to speculation that the ingestion of these fibres might be associated with an increased risk of gastrointestinal cancer in the general population; the realisation that many beverages and drugs contained asbestos fibres (22, 142), and that asbestos cement piping was widely used for water supplies, provided additional impetus for research into the potential hazards of asbestos ingestion. The present study was undertaken as part of that research.

A preliminary report (51) has shown that continuous exposure of rats to ingested asbestos for periods up to one year was not associated with any significant widespread damage or evidence of fibres penetrating the gastrointestinal mucosal tissues, despite the rats being given about 50 000 times the calculated likely human maximum (630); a continuation of the study to 130 weeks has confirmed these findings. However, it is possible that the ingestion of asbestos is associated with subtle changes in cell proliferation of the gastrointestinal mucosa, since force feeding of large amounts of asbestos has

been shown to cause a temporary increase in mucosal proliferation as measured by the uptake of tritiated thymidine (22, 23). We shall describe experiments in which we examined mucosa from selected gastrointestinal sites in rats exposed to a more realistic dose regimen of continual asbestos ingestion, which was nevertheless many thousands of times higher than the calculated human maximum ingestion.

5.5.2 MATERIALS AND METHODS

Male Wistar rats, of the AF/HAN strain, ten weeks old at the start of the study, were used throughout. They were housed two per cage and maintained on standard pelleted laboratory diet. Eight treated animals were given free access to a dietary supplement of margarine containing 5 mg amosite asbestos per g. Amosite was chosen because it is most similar to the contaminating fibres in Canadian drinking water, and the UICC standard reference sample was used (572, 716); the asbestos was administered in margarine to prevent the generation of airborne fibres. Five positive control rats received the margarine supplement without amosite, and eight negative controls received only the normal diet. The animals given margarine consumed 50 to 60 g per week, corresponding to between 250 and 300 mg of asbestos fibre for those animals given the margarine-amosite formulation.

The cytokinetic analyses were performed when the animals were 120 weeks old. Two approaches were used; the first procedure involved estimating the amount of tritiated thymidine (^{3}HTdR) incorporated by the gastrointestinal tissues, using liquid scintillation counting (22); the second employed autoradiography to obtain the pattern of intestinal crypt cell proliferation, and a stathmokinetic determination of the cell birth rate. Animals were injected with either 0.5 μCi ^{3}HTdR per g body weight (three amosite-treated and three negative control), or 1 μg vincristine sulphate per g body weight (five from each treatment group), all injections being performed at 0900 h to try to avoid the effects of diurnal variability in proliferative activity (652). Animals were killed by cervical dislocation 60 minutes after ^{3}HTdR, and 30, 60, 90, 120 and 150 minutes after vincristine.

The upper, mid, and lower small-intestinal tissue samples were taken from the first 10 cm, the region between 35 and 45 cm, and the distal 10 cm respectively, of the excised small intestine. Similarly, the ascending and descending colon samples were taken from the first 5 cm and the region between 15 and 20 cm of the excised colon.

5.5.2.1 DNA EXTRACTION AND ASSAY

Immediately after death the rats given ^{3}HTdR were exsanguinated and samples of glandular stomach, upper, middle and lower small intestine, caecum, ascending and descending colon, liver and spleen were immersed in liquid nitrogen and then stored at −70°C. Subsamples of 100 mg wet weight were used for the assays, care being taken to avoid the Peyer's patches in the samples of small-intestinal tissues, and these subsamples were homogenised in cold 10 percent trichloroacetic acid (TCA) using a motor-driven teflon pestle and glass tubes. After the homogenate had stood over ice for 20 minutes, the precipitate was collected, washed twice with cold 95 percent ethanol and twice with one part ethanol to three parts ether; then it was centrifuged, collected again, resuspended in cold 6 percent TCA, and divided into two equal parts. One half was used for the radioactivity estimations by liquid scintillation counting: the precipitate was recovered from the TCA, and dissolved in 1 N NaOH at 90 °C; then the solution was neutralised with 6 N HCl and bleached with hydrogen peroxide. Duplicate samples of the preparation were mixed with NE260 scintillant and counted at 4 °C after overnight equilibration. Full quench calibrations were prepared, and the activity of the tissue samples expressed as mean disintegrations per minute (dpm). The other half of the precipitate was used for total DNA estimations: it was hydrolysed in 6 percent perchloric acid at 100 °C and triplicate samples of the hydrolysate were analysed using Burton's modification of the diphenylamine assay for DNA (76) with calf thymus DNA standard.

Three 100 mg samples of each tissue were processed for each rat and the mean activities (in dpm per μg DNA) in treated and control groups were compared using analysis of variance.

5.5.2.2 CYTOKINETIC ESTIMATIONS

Tissues from the three small-intestinal sites and from the descending colon were fixed in cold Carnoy's fluid for six hours and embedded in paraffin wax. Histological sections 3 μm thick were stained with Harris's haematoxylin and eosin. For those rats given ^{3}HTdR, autoradiographs were prepared by a dipping technique and exposed for two weeks. From each sample of tissue 100 axially-sectioned crypts were analysed (82, 818) and from transversely sectioned crypts the column count and Tannock's factor (702) were estimated. The product of the mean crypt length and column count was taken to be an estimate of the total number of cells per crypt, and the slope of the line of best fit to the data relating corrected mitotic index to time after vincristine administration (from 30 minutes onward) was multiplied by this total to give an estimate of the crypt cell production rate (CCPR). The cut-off position, at which the average cell begins its final cycle, was also calculated (28, 84).

5.5.3 RESULTS

The animals used for these investigations were 120 weeks old at the time of assay, and as such displayed some of the usual age-associated pathology (72). The renal hypertensive tubular damage frequently described in aged rats was present to some degree in all animals, and those given access to margarine were obese, having a mean body weight of 720 g compared to 510 g for the negative controls, and showed widespread hepatic periportal fatty change. None of the animals had any specific pathological lesion which could be considered to have had a major effect on gastrointestinal cell proliferation.

5.5.3.1 DNA ESTIMATIONS

Table 5.5.1 summarises the results of the DNA estimations for the amosite-

Table 5.5.1 A measure of DNA-synthetic activity in various tissues in amosite-treated and negative control rats. Units are dpm/μg of DNA, and numbers given are the means of triplicate estimates on each of three rats

Tissue	Control	Amosite
Glandular stomach	80	59
Upper small intestine	370	370
Mid small intestine	590	670
Lower small intestine	190	200
Caecum	510	610
Ascending colon	180	180
Descending colon	220	150
Liver	23	37
Spleen	150	180

treated rats and the negative controls. Only in the descending colon are the two counts significantly different ($F_{1,4} = 35.48$; $p < 0.01$), where the treated animals incorporated less labelled thymidine than the controls, suggesting a depression in proliferative activity associated with the ingestion of asbestos and margarine.

5.5.3.2 MORPHOMETRIC AND CYTOKINETIC ESTIMATIONS

Table 5.5.2 summarises the dimensions of the crypts at different sites in those animals used for stathmokinetic experiments; there was one anomaly in that the crypts of the descending colon in the five rats used for labelling index estimation were on average some 10 cells longer than in those which took part

Table 5.5.2 Values of morphometric and cytokinetic variables in the crypts at different sites in the gut of amosite-treated and negative-control 120-week-old rats

	Treatment	Upper small intestine	Mid small intestine	Lower small intestine	Descending colon
Crypt length (cells)	Control	36.0	32.1	31.8	41.8
	Amosite	35.7	32.6	31.4	41.1
Column count	Control	22.0	20.1	21.1	20.8
	Amosite	20.0	20.8	20.4	20.2
Total cells per crypt	Control	790	650	670	870
	Amosite	710	680	640	830
Corrected mitotic index (%)	Control	1.95	2.02	2.06	2.13
	Amosite	2.50	2.21	1.92	1.10
Labelling index (%)	Control	26.9	25.0	27.2	12.5
	Amosite	28.3	26.8	25.2	8.7
CCPR (cells/crypt/h)	Control	39	26	28	24
	Amosite	40	45	22	13

in the stathmokinetic experiment. There were no significant differences in crypt size at any site between control and treated rats. The table also shows the mitotic and labelling indices for the whole crypt, and the cell birth rate; the only consistent difference is the apparently decreased proliferative activity in the descending colon of the treated rats. Figure 5.5.1 illustrates the labelling index at each cell position at that site in the two groups.

Table 5.5.3 gives further data for the descending colon, and includes results for the margarine-fed controls; also presented are comparative results for 12 week old rats (688). Because of the small numbers of animals available, the birth rate estimates have rather large standard errors, being some 25 percent of

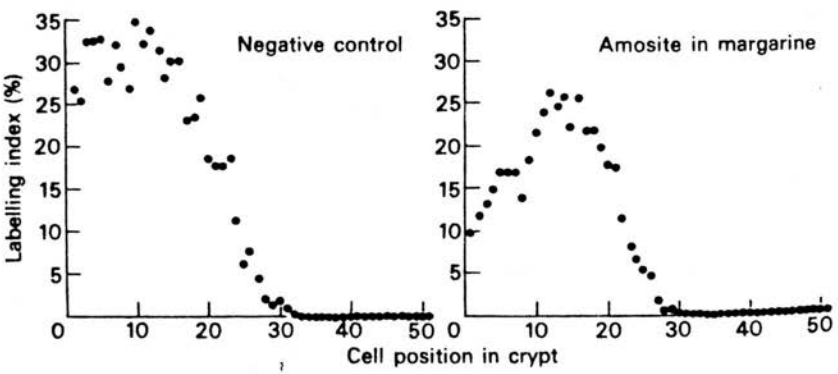

Figure 5.5.1 Labelling index distributions in the descending colon of (a) control rats and (b) rats given amosite in margarine supplement

Table 5.5.3 A comparison of variables in the descending colon of differently treated 120-week-old rats and 12-week controls (688). Cell cycle times are based on a constant growth fraction estimate of 0.34 (638)

Treatment	Crypt length (cells)	Cut-off position	Birth rate (cells/crypt/h)	Cell cycle time (h)
Amosite in margarine	41	11	13	23
Margarine-fed controls	39	10	9	29
Negative controls	42	8	24	12
12-week controls	42	11	7	34

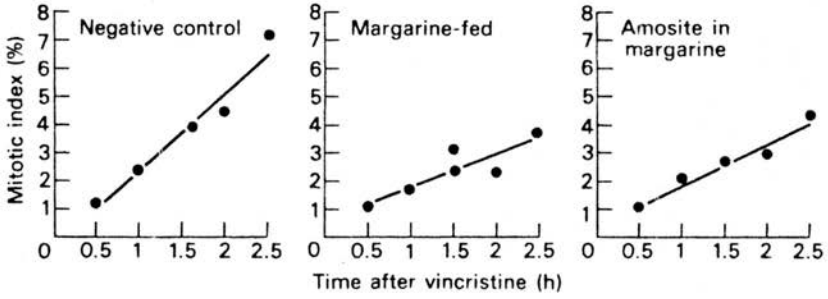

Figure 5.5.2 Mitotic accumulation data after vincristine administration in the descending colon of (a) rats given a normal diet, (b) rats given margarine supplement, and (c) rats given amosite in margarine supplement

the estimates themselves; Figure 5.5.2 shows the metaphase accumulation data which gave rise to them.

5.5.4 DISCUSSION

There was no clear-cut effect of prolonged ingestion of asbestos on cell proliferation in the rat intestine. The findings of the DNA extractions and the cytokinetic analyses were similar, confirming that bulk estimation of the extent of DNA synthesis can provide useful information on proliferative activity. It is interesting that both techniques showed a depression in activity in the descending colon of amosite-treated animals compared to negative controls, but further comparison with margarine-fed controls suggests that this is more likely to be associated with the high intake of margarine than with asbestos fibre ingestion; indeed it is the high cell birth rate in the negative-control rats which is difficult to explain, as it is considerably in excess of that found in younger

animals (see Table 5.5.3). It is not clear whether a depressive effect of a high fat diet should be attributed to reduction in cell attrition arising from changes in food transit time, to the composition of either the luminal contents or the intestinal flora, or alternatively to some direct regulatory influence as yet unknown.

Although ingestion of asbestos has been shown to be associated with an increase in gastrointestinal DNA synthesis (22, 23, 347, 348), these findings were concerned with the effects of a challenge with massive doses of the more toxic chrysotile form of asbestos, and may be attributable to a combination of cytotoxicity and the sort of alteration to regulatory mechanisms of proliferation which is known to occur in response to such non-specific stimuli as changes in intestinal flora (2) or secretions (7), starvation and refeeding (18, 110), parasitic infection (431) and diet (409). If one may draw such conclusions from animal experiments, the absence of any adverse effect of amosite ingestion previously reported (51), taken with our current findings, suggests that low levels of asbestos fibre contamination in food, beverages, and drinking water are unlikely to have any major significance for the health of the general population.

The present studies are of a more general interest for their observations on the proliferative characteristics of gastrointestinal tissues of aged rats as such. Under normal circumstances the male SPF Wistar rat of the HAN strain may live for up to three years, with 80 percent mortality occurring by about 130 weeks. The animals used in this study were 120 weeks old and therefore represent a survivor population of aged animals. A detailed description of the morbidity and mortality of the animals exposed to chronic asbestos ingestion will be the subject of a future paper.

It has been suggested that intestinal proliferative homeostasis is less effective in aged mice and that proliferative activity is reduced as a result of a decrease in the size of the proliferation compartment in the crypt (241, 414). A study of rats at several ages up to a year old (116) demonstrated an increase with age in the ratio of crypts to villi, but little change within the crypts themselves. A comparison of our results in the descending colon shows that there is probably little change in the size of the crypt, and leaves us uncertain about how age affects crypt cell production rate. In the small intestine the picture is clearer: we may compare our results (Table 5.5.2) with those previously published (6, 814, 818) for rat jejunum. Our crypt length and column count are not far from the 32.9 and 22.3 cells respectively given in these reports, and our corrected mitotic index, labelling index and crypt cell production rate are all rather less than the respective values of 3.1 percent and 57 cells per crypt per hour for three-month-old animals. This is consistent with a decrease in the size of the proliferation compartment in the aged rats: in fact the cell position at which the labelling index is half of its maximum is some three or four positions lower in the crypt than for younger rats, implying a cut off position a cell or two lower. However, assuming a cell cycle time of 10.9 h over the relevant part of the

crypt (6) we can estimate the cut-off to occur at cell position 12 from data on all three small-intestinal sites in the aged amosite-treated and control rats, and at position 11 in three-month-old animals. It therefore remains a possibility that the cell cycle time in the older rats is longer; but if this is not to result in an upward movement of the position of half-maximum labelling index it must be brought about by an increase in the duration of G_2.

We do not consider that we have enough data to assert that G_2 is indeed increased, but comparison of the phase duration in the same strain of rats of different ages by a single worker would be of interest.

ACKNOWLEDGEMENTS

We would like to thank Professor Sir Alastair Currie and Dr D Lamb for the use of the facilities of the Pathology Department of the University of Edinburgh. Thanks are also due to Dr N A Wright for his helpful suggestions in the earlier phase of this work.

Cell Tissue Kinet. (1984) **17**, 445–452.

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Morphometric and kinetic studies on the changes induced in the intestinal mucosa of rats by intraperitoneal administration of quinacrine

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(*Received* 13 *September* 1983; *revision accepted* 21 *February* 1984)

Abstract: The intraperitoneal administration of large doses of quinacrine in rats results in a state of enteromegaly affecting mainly the distal small bowel, caecum and proximal colon. This enteromegaly is associated with mucosal crypt hyperplasia, and hypertrophy and hyperplasia of the *Muscularis propria.* In order to investigate the changes in the intestinal mucosal crypts, morphometry and a metaphase-arrest experiment with vincristine were undertaken on a group of rats given 12 mg of quinacrine hydrochloride by intraperitoneal injection daily for 5 days 2 weeks previously, and comparisons drawn with a group of control animals.

In the quinacrine-treated animals there was marked enteromegaly affecting the distal small bowel, caecum and proximal colon, and in these segments there was clear crypt hyperplasia. Proximal and distal to the dilated bowel hyperplasia was not seen. No consistent pattern of change in crypt-cell birth rate was evident. The mechanisms by which quinacrine may effect kinetic and morphometric changes in the intestinal crypts are considered.

Quinacrine (mepacrine, Atabrine, 2-chloro-5-(4-diethylamino-1-methylbutylamino)-7-methoxyacridine dihydrochloride) is a yellow acridine dye which fluoresces under ultraviolet light. Formerly it was extensively used in the treatment and suppression of malaria, but it has now been superseded by antimalarial agents which are more effective and less likely to cause side-effects. The drug is still used clinically however, in the treatment of giardiasis and in tapeworm infestations. The clinical usefulness of the drug is likely to be due to its ability to bind to nucleic acids (Albert, 1965). Side-effects are quite common in man and include skin pigmentation, rashes, gastrointestinal disturbances and CNS effects. They are usually mild, however, and even in massive overdosage the drug is relatively non-toxic (Wade, 1977).

The intraperitoneal (i.p.) administration of high doses of quinacrine to rats causes enteromegaly and distension of the bowel, affecting principally the intestine from mid-small bowel to proximal colon. It also causes steatorrhoea (Keeler, Richardson & Watson, 1966). The drug has been shown to inhibit the contractile activity of the smooth muscle of the gut wall, probably due to toxic effects on its innervation (Smith, 1972), thus inhibiting peristalsis. This results in impaired intestinal transit and the build-up of intestinal contents, particularly conspicuous in the ileum, caecum and ascending colon. In addition to intestinal dilatation, a

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further prominent feature is the thickening of the bowel wall which occurs at affected sites. This thickening is reminiscent of the adaptive changes which occur in the remaining small bowel after subtotal small-intestinal resection (Senior *et al.*, 1982); and preliminary morphological investigations have suggested that the muscular hypertrophy may be accompanied by a state of mucosal crypt hyperplasia (Sunter, unpublished observations). It is the purpose of this communication to investigate the morphometric and kinetic changes induced in the intestinal mucosal crypts of rats treated with quinacrine, and to determine the nature and extent of the crypt hyperplasia.

MATERIALS AND METHODS

Female Wistar rats were used throughout. The animals were obtained from Olac Ltd, Bicester, at the age of 10–12 weeks. They were housed in cages of six and maintained on a normal animal house routine. They were fed on No. 3 expanded rat and mouse breeder's diet (SDS, Witham) and were allowed tap water *ad libitum*.

The experiments were performed when the animals were 24 weeks of age. Those animals in the experimental group were given 12 mg of quinacrine hydrochloride (BDH Chemicals, Poole) as 1·6 ml of a 7·5 g/l aqueous solution intraperitoneally on each day for 5 days. The control animals were given a similar 5-day course of injections of the same volume of 0·9 g/l saline solution.

Two weeks after the final quinacrine injection ten experimental and ten control animals were given vincristine sulphate ('Oncovin', Eli Lilly, Basingstoke) by i.p. injection at a dosage of 1 mg/kg body weight (BW). These injections were administered at 0900 hr and at 15-min intervals thereafter animals were killed *seriatim* by ether overdosage. Post-mortem dissection was performed as described below.

On the same day that the vincristine experiment was carried out, three experimental and three control animals were given an i.p. injection of tritiated thymidine ([^{3}H]TdR) (Amersham International, Amersham), consisting of 1 mCi/kg BW, the specific activity of the [^{3}H]TdR being 5 mCi/mmol. These animals were killed and dissected 1 hr later (see below).

Immediately *post mortem*, blood was taken by cardiac puncture from six treated animals and from four controls. This was used to assess liver function by the determination of serum bilirubin, alkaline phosphatase and aspartate transaminase (AST), using a Cobas bioanalyser.

The general post-mortem examination performed on all animals included visual inspection of all viscera, and blocks were taken for histology from the liver, kidneys, spleen and lungs of all experimental animals and from a representative sample of controls. These blocks were fixed in neutral-buffered 4% formal saline. The entire intestine from the pylorus to the anus was removed and then divided at the ileo–caecal junction. The small bowel was fixed unopened in Carnoy's solution; the large bowel was opened along its length, pinned out on a cork board mucosal surface uppermost and then fixed in a bath of Carnoy's solution. Following 12–18 hr fixation the intestines were transferred to cellosolve (2-ethoxyethanol) and subsequently transverse blocks were taken for microscopy from the following sites: (a) proximal small bowel, a site 20 mm distal to the pylorus; (b) mid-small bowel, from the midpoint between the pylorus and the ileo-caecal junction; (c) distal small bowel, a site 30 mm proximal to the ileo–caecal junction; (d) proximal colon, a site 90% of the distance between the anus and the colo-caecal junction; and (e) distal colon, a site 30% of that distance.

The formalin-fixed material taken for routine microscopy was processed in the usual way to paraffin wax, sections were cut at 5 μm and stained with haematoxylin and eosin, other

staining procedures being used in addition as necessary. The blocks of intestine for quantitative microscopy were dehydrated in changes of cellosolve and then transferred to chloroform; thereafter they were embedded in a semisynthetic wax ('Polywax', Difco, West Molesey) and serial vertical sections produced at a thickness of 3–4 μm. The sections from the vincristine experiments were then stained by haematoxylin and eosin. Those from the TdR-labelled animals were subjected to autoradiography using Kodak AR 10 stripping film. Following a 4-week exposure period they were developed and fixed and then stained by Harris' haematoxylin and mounted. Prior to assessment the slides were randomized to eliminate observer bias. A qualitative assessment of all material was made first, then a quantitative analysis of the state of the intestine was performed.

One hundred perfect axial sections of mucosal crypts were counted for each block. The length of each crypt (in cells) was recorded, counting from the most basal cell (cell position 1) to the crypt–villus junction in the case of the small bowel and to the crypt–surface junction in the case of the large bowel. The cell positions of arrested metaphase figures were also recorded. The absence of any post-metaphase mitotic figures confirmed the completeness of metaphase arrest. Four blocks from each site in the bowel for both control and treated animals were then turned through 90° and histological cross-sections of crypts produced and stained. From fifty cross-sections of crypts in each block the crypt circumference (in cells), the column count, was counted and an assessment of Tannock's factor (Tannock, 1967) made.

Mean crypt lengths at the various sites in the bowel in the two groups of animals were calculated, and the accumulation of arrested metaphases was plotted against time after vincristine over the 150-min collection period (Wright & Appleton, 1980). From the slope of the fitted line a value for the birth rate (K_B) and its standard error were calculated.

RESULTS

Gross pathological findings

Shortly after the first injection the treated rats became listless and their appetite was reduced. By the end of the treatment schedule these symptoms were more obvious and abdominal distension had become apparent in some of the animals. In the 2-week period prior to the time the kinetic experiments were performed, all the quinacrine-treated animals developed marked abdominal distension. Mortality was high with 30% of the animals either dying spontaneously or having to be killed for humane reasons in the period commencing 3 days after cessation of treatment. This loss was uniform over the post-treatment period and not obviously related to the degree of abdominal distension; it was much greater than that previously observed (Keeler *et al.*, 1966; Sunter, unpublished observations).

At autopsy all treated animals were emaciated with virtually a complete loss of the usually abundant depot fat. A somewhat unexpected finding was the presence of bloodstained ascitic fluid in the peritoneal cavity, often as much as 10–15 ml in volume. But morbidity and mortality did not appear to be related to the quantity of fluid present. Gross examination of the intestine revealed a variable degree of distension and muscular hypertrophy affecting mainly the mid-small bowel, distal small bowel, caecum and proximal colon. In some animals the affected bowel was several times the normal calibre. The luminal contents were rather more viscous than normal and of a faecal nature. The small intestine was considerably shortened in all animals and this shortening appeared to involve specifically those segments of distal jejunum and ileum, the site of the greatest thickening. In some animals the small bowel

was reduced to as little as half the normal length. Shortening of the colon was in contrast not conspicuous, even when dilatation of the proximal colon was considerable.

Inspection of the other internal viscera in treated animals showed no other abnormalities. Macroscopic findings in the control animals were not unusual.

Microscopical findings

Examination of the kidneys and spleen showed no significant abnormalities in either treated or control rats. The lungs of both groups showed those granulomatous lesions which are considered a normal finding in rats (Innes, 1965, 1967). In the control animals, hepatic abnormalities were present consisting of a variable focal mononuclear infiltrate in portal areas with occasional small epithelioid granulomata distributed both within portal areas and within hepatic parenchyma. This non-specific hepatitis was much less conspicuous in quinacrine-treated rats, and although of obscure cause may thus be related to some state of infestation. Hepatic abnormalities induced by quinacrine included focal fatty change, focal inflammation and focal regenerative activity. Serum bilirubin levels were similar in control and treated animals, but the values of serum alkaline phosphatase and AST were higher in control animals, probably a reflection of their mild chronic hepatitis.

In those sites in the intestine which appeared abnormal in the quinacrine-treated animals, microscopy confirmed the presence of marked hypertrophy affecting both the *Muscularis propria* and the *Muscularis mucosae*. There was, in addition, evidence of muscular hyperplasia, there being mitotic activity clearly recognizable in smooth muscle cells. The mucosa at these sites was also thickened, but no evidence of any other mucosal abnormality—e.g. inflammation or ulceration—was seen. There was crypt hyperplasia (see below) but the intestinal epithelium was morphologically normal, with no suggestion of dysplastic or other alteration. A mild chronic fibrinous peritonitis was also in evidence.

Although the autoradiographs from the control animals demonstrated perfectly adequate labelling of crypt-cell nuclei, those from the treated animals showed a complete absence of nuclear labelling. Labelling also failed in a further cohort of thirty quinacrine-treated animals given i.p. [^{3}H]TdR as part of another experiment, not described here, performed at the same time as the present study. This suggests that simple misplacement of the injected solution is unlikely to be the reason for the problem and the precise cause for this discrepancy is a matter of speculation; the presence of ascitic fluid may have interfered with [^{3}H]TdR absorption from the peritoneum or abnormalities of the peritoneum itself may have prevented absorption. Alternatively it is possible that enzymes in the ascitic fluid degraded the [^{3}H]TdR prior to its absorption. This latter possibility is perhaps most likely since careful scrutiny of the sections from the vincristine experiment showed adequacy of metaphase arrest 30 min after vincristine administration, confirming that at least the vincristine was being absorbed from the peritoneum satisfactorily. Clearly an alternative route of [^{3}H]TdR administration is needed—e.g. intravenously—if labelling experiments are to be performed successfully on quinacrine-treated animals.

Morphometric and cytokinetic findings

The morphometric findings are summarized in Table 1. In the three sites where distension of the intestine was greatest, viz. mid-small bowel, distal small bowel and proximal colon, there was clear evidence of crypt hyperplasia, with an increase in crypt length but little alteration in column count. In the proximal small bowel and distal colon, sites showing no evidence of dilatation, crypt lengths were unaltered, but there appeared to be a slight reduction in crypt population size due to a fall in column count.

Table 1. Morphometric data for crypts at several sites in the intestines of control and quinacrine-treated rats

	Proximal small bowel		Mid-small bowel		Distal small bowel		Proximal colon		Distal colon	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Crypt length (cells)	31·3 (±0·62)	32·2 (±0·68)	24·7 (±0·59)	31·0** (±0·61)	24·0 (±0·50)	30·6** (±0·36)	20·0 (±0·24)	27·1** (±0·63)	29·8 (±0·58)	30·8 (±0·59)
Column count (cells)	20·1 (±0·77)	17·8 (±0·30)	18·1 (±0·11)	19·1 (±0·67)	20·6 (±0·38)	18·4** (±0·18)	18·2 (±0·88)	16·1 (±0·52)	19·1 (±0·79)	15·7** (±0·22)
Crypt pop. (cells)	629 (±27)	573 (±15)	447 (±11)	592** (±24)	494 (±14)	562** (±9)	364 (±18)	436* (±17)	569 (±26)	484* (±11)

Mean values are given in cells, with standard errors in parenthesis; * indicates a statistically significant difference ($P < 0{\cdot}01$), and ** indicates a statistically significant difference ($P < 0{\cdot}001$). Since calculation of crypt population from the product of crypt length and column count leads to a slight overestimate, the standard errors should be regarded with caution.

Table 2. Crypt-cell birth rates and crypt-cell production rates (± SE) and relative proliferation zone sizes (RPZS) at several sites in the intestines of control and quinacrine-treated rats

	Proximal small bowel		Mid-small bowel		Distal small bowel		Proximal colon		Distal colon	
Group	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Birth rate (cells/1000/hr)	47·6 (±5·8)	37·1 (±3·5)	39·5 (±2·4)	39·0 (±8·0)	44·7 (±5·8)	22·4* (±4·0)	7·3 (±1·1)	8·6 (±2·6)	8·2 (±1·9)	3·2 (±3·0)
CCPR (cells/1000/hr)	29·9 (±3·9)	21·0 (±2·1)	17·7 (±1·2)	23·1 (±4·8)	22·1 (±2·9)	12·6* (±2·3)	2·7 (±0·4)	3·8 (±1·2)	4·7 (±1·1)	1·5 (±1·4)
RPZS	0·7	0·68	0·77	0·74	0·75	0·72	0·7	0·59	0·59	0·58

* indicates a statistically significant difference ($P < 0{\cdot}01$). The standard errors of CCPR are again subject to the limitations imposed by the method of calculating crypt population sizes.

Table 2 shows results from the stathmokinetic experiment for the several sites along the length of the bowel. Birth rates were reduced (though not significantly) in the somewhat hypoplastic crypts in the non-dilated segments of the intestine in the quinacrine-treated animals. In the dilated segments they were either little changed or markedly reduced and no consistent pattern emerges. The product of crude cell birth rate and crypt population size gives an estimate of the rate of efflux of cells per crypt—the crypt-cell production rate (CCPR). These values are given in Table 2. The hypoplastic crypts of proximal small bowel and distal colon show marked reductions in CCPR, but in the dilated segments of the bowel changes are inconstant. Estimates of the size of the proliferative zone within the crypt (Appleton *et al.*, 1980) are also given in Table 2. They show no convincing changes.

DISCUSSION

These results confirm previous observations of abdominal distension and enteromegaly in rats given several large doses of quinacrine intraperitoneally (Keeler *et al.*, 1966). While administration of a single large dose has been shown to produce reversible changes (Nair, Munshi & Devi, 1977) as do multiple smaller doses (Smith, 1967, 1972), the unexpectedly

severe morbidity and high mortality in the present animals suggest far more profound effects in this strain. The bloodstained ascites is a feature not previously commented on and may indicate a significant difference in the sensitivity of some strains of rat to the drug.

Precise data regarding food intake were not obtained in either control or treated animals but it was obvious that the quinacrine-treated animals were eating much less than controls and became emaciated. And while the presence of a variable amount of ascitic fluid in treated rats, together with a variable but considerable accumulation of faecal material in the distended segments of gut, make a simple comparison of the body weights of treated and control animals of doubtful value, the virtually complete absence of depot fat in treated animals indicates that they have undergone severe nutritional deprivation. Presumably this is due to abnormalities of gastrointestinal motility as well as anorexia. Despite this state of 'starvation', crypt population sizes were only slightly reduced in the non-dilated segments of the intestine, and were actually increased in the dilated segments. This situation contrasts with that in animals which have actually been subjected to simple starvation (Al-Dewachi *et al.*, 1975), when generalized crypt atrophy is seen, and in segments of bowel subject to stasis by isolation from the nutrient stream, when mucosal atrophy is the rule (Gleeson, Cullen & Dowling, 1972), although it should be noted that in self-filling small-intestinal loops a state of mucosal hyperplasia supervenes (Menge *et al.*, 1979). It is possible therefore that local trophic influences from the lumen of the bowel may be responsible for masking the hypoplastic response to functional starvation in this model. Indeed in the most dilated segments of the bowel crypts are largest. The effect is unlikely to be purely a mechanical one since simply increasing faecal bulk has no effect on crypt population size (Dowling *et al.*, 1967), and the production of intestinal stasis and distension by colonic ligation does not produce small-intestinal hypertrophy (Keeler *et al.*, 1966; Burnham, 1983), although this latter author showed clear colonic mucosal hyperplasia. Thus it would appear unlikely that local trophic influences are the only ones which are preventing crypt atrophy in these nutritionally deprived animals, and it is possible that some direct effect of the drug is important.

The results of the stathmokinetic experiment are difficult to interpret. In the two sites which were not affected by gross distension—i.e. the proximal small bowel and the distal colon—both cell birth rate and CCPR were reduced. These changes, along with the reduced crypt-cell populations, are possibly related to the general state of functional starvation (Al-Dewachi *et al.*, 1975). But despite the changes, crypt length was maintained, in contrast with the situation in experimental starvation (Al-Dewachi *et al.*, 1975) and in parenteral feeding (Levine *et al.*, 1974). At the three sites where gross distension was found there was actually crypt enlargement. But consistent changes in birth rate and CCPR were not evident, CCPR being increased in the mid-small bowel and proximal colon but diminished in the distal small bowel. This inconsistency is difficult to explain, but it is possible that in the terminal portion of the ileum there is some site-specific antitrophic effect occurring at a local level.

Administered in high doses quinacrine stains DNA (Albert, 1965; Smith, 1967, 1972) but DNA synthesis is not impaired (Lerman, 1961). In low doses it is taken up into nerve cells, particularly those in Auerbach's plexus and those innervating intestinal smooth muscle (Smith, 1967, 1972; Alund & Olsen, 1980). The affected nerves are non-adrenergic and non-cholinergic (Alund & Olsen, 1980) and may be purinergic or peptidinergic in nature. There are a large number of peptidinergic nerves present in intestine (Alund & Olsen, 1979). Of particular interest in the context of the present study, and especially in view of current interest in the role of gut endocrine cells on intestinal cell renewal and adaptive responses, is the observation that the drug concentrates in some but not all enteroendocrine cells. Uptake into

the gastrin-secreting cell has been demonstrated, and other cell types not yet characterized also stain. In view of the suggestion that enteroglucagon may function as the gut trophic hormone it may be significant that the pancreatic glucagon cell is also stained by quinacrine (Ekelund *et al.*, 1980). Thus there may exist a link between the *in-vivo* distribution of the drug in neuronal and enteroendocrine cells of different types, and the distribution of the morphometric and kinetic changes. The changes observed were, however, obtained using very high doses of quinacrine and it remains to be seen whether similar types of change can be induced by smaller dose levels, and whether there is any correlation between the degree of response and changes in circulating or local tissue levels of putative trophic hormones, such as enteroglucagon.

ACKNOWLEDGMENTS

This work was supported by grants from the Northern Council of the Cancer Campaign. We would like to thank Mrs K. Elliot for technical assistance and Miss A. Lott who typed the manuscript.

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Stathmokinetic studies on intestinal crypts following 70% resection of the small bowel or transplantation of 10–20 mm of small bowel to the exterior abdominal wall

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INTRODUCTION

Following resection of a major portion of the small bowel in experimental animals, adaptive changes occur affecting all layers of the remaining intestine; these result in dilatation, thickening and lengthening of the gut together with hyperplasia of the crypt-villus system[1-6]. The severity of these changes is related to the amount of intestine removed and they are most prominent immediately distal to the anastomosis. Expansion of the proliferation compartments of the crypts in this post-anastomotic area of the small bowel has been reported[3,4,7-9] together with slight shortening of the cell cycle time[7-9]. This adaptive process results in the production and maintenance of an increased population of absorptive cells, which compensates for the loss of intestine[10].

Operative procedures of this type have been used in attempts to elucidate the mechanisms governing the production of epithelial cells under normal and pathological conditions. Proximal resection of the small bowel exposes the remaining gut to abnormally high concentrations of certain nutrients which may act topically to stimulate hyperplasia[1,11] and to raised levels of gastric, duodenal and pancreaticobiliary secretions[12,13], the latter being most trophic[14,15]. Chalone-like substances have been extracted from both the large bowel[16] and the small bowel[17] and these may play a part in intestinal adaptation. There is also evidence from parabiosis experiments for humoral stimulation[18], though the existence of such influences has been disputed[19]. The role of hormones[20-25] and of the autonomic nervous system[26] have also

been investigated. The relative importance of these various factors has recently been reviewed by Williamson[27-29].

Loehry and Grace[30] have described an operative procedure whereby a segment of small bowel is brought through the abdominal wall on a vascular pedicle, opened and sutured to the skin. This abnormal environment, which also involves exposure to mechanical trauma, stimulates intense crypt hyperplasia. In the present study we have studied the effects of 70% proximal resection, and of exteriorization of a segment of small bowel, on the cytokinetics of the mucosal crypts. The significance of our findings is discussed in the context of the control of intestinal adaptation.

MATERIALS AND METHODS

Surgical procedures

Female Wistar Porton rats between 20 and 23 weeks old and weighing between 170 and 210 g were used for both surgical procedures. Anaesthesia was induced with ether, followed by 0.2 ml/kg pentobarbitone sodium (Sagital, May & Baker) administered intraperitoneally; anaesthesia was maintained with ether during surgery. For both procedures the abdomen was opened with a 30–40 mm midline incision and the intestine was then gently exteriorized and rotated to facilitate identification of small bowel anatomy. One or other of the following operations was then performed.

70% Resection

A mesenteric vascular arcade 100–110 mm proximal to the caecum was chosen and the mesentery between the blood vessels was split with fine scissors. The ligament of Treitz was identified and the arcade immediately distal to it similarly treated. The branches of the superior mesenteric blood vessels between these two positions were tied off in two bunches with 4–O silk suture (Ethicon); the bowel was divided proximally and distally and the intervening intestine removed. The cut ends were opposed and an end-to-end anastomosis was fashioned using a continuous single layer of 8–O silk suture with additional stitches as required. The intestine was replaced and 2–3 ml of normal saline were introduced into the peritoneal cavity. The abdominal wound was closed with two layers of interrupted 4–O silk suture.

Exteriorization of a patch of small bowel

A mesenteric vascular arcade 100–120 mm proximal to the caecum was chosen and a segment of intestine 10–20 mm long was mobilized on a vascular pedicle. Intestinal continuity was restored by end-to-end anastomosis as described above. The mobilized segment of bowel was brought through the abdominal incision which was closed around the pedicle with interrupted 4–O silk sutures, care being taken not to compromise

the blood supply by twisting or traumatizing the pedicle. The segment of bowel was opened and the mucosa cleaned. The skin was then trimmed to the correct shape to receive the patch whose corners were first anchored to the skin with single stitches before sewing it into place with a continuous single layer of 6–O silk suture.

Other experimental methods

Food was withheld for 24 h post-operatively, after which standard rat pellets (Breeders Diet, BP Nutrition) were supplied *ad libitum*. Seven weeks after the operations 9 patch-bearing and 10 resection animals were given a single i.p. injection of vincristine sulphate (Oncovin, Eli Lilly) at a dose of 1 mg/kg body weight at 9 a.m. Animals were then killed by ether overdose at 15 min intervals commencing 30 min after injection for the 'patch' animals and 15 min after injection for the 'resection' animals. The whole bowel was removed and divided at the ileocaecal junction. The exteriorized patch together with a small amount of skin was trimmed free and fixed. The small bowel was fixed intact; the large bowel was opened and pinned to a cork board prior to fixation. All tissues were fixed in Carnoy's fluid for 12–18 h before transfer to Cellosolve. Tissue blocks for histology were taken from the following sites in the intestine (see also Figure 32.1):

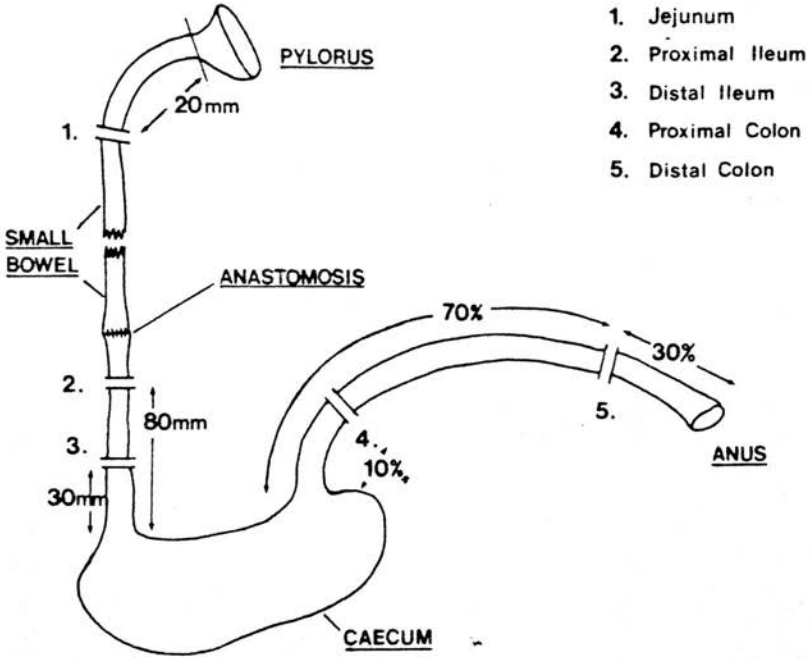

Figure 32.1 Sites in the bowel from which blocks for histology were taken

(1) jejunum 20 mm beyond the pylorus, i.e. proximal to line of resection;
(2) proximal ileum 80 mm from the ileocaecal junction, i.e. distal to line of resection;
(3) distal ileum 30 mm from the ileocaecal junction;
(4) proximal colon 10% of the distance from the caecum to the anus;
(5) distal colon 70% of the distance from the caecum to the anus;
(6) the centre of each exteriorized patch.

Blocks were also taken across the line of resection and from the skin-patch junction to assess healing.

After processing and embedding in Polywax (Difco), vertical 3 μm thick histological sections were cut and stained with haematoxylin for morphological evaluation and analysis.

The number of cells in the 'left-hand' side of 100 well-orientated crypt profiles from each block (the crypt length) was recorded together with the number and position of every arrested metaphase (Figure 32.2). To evaluate the column count, i.e. the number of cells per crypt circumference, blocks from each site from the animals killed at 105, 120, 135 and 150 min after vincristine were turned through 90° to provide histological cross-sections of the crypts (Figure 32.2). The number of cells per crypt circumference from 50 crypts per time point, a total of 200 crypts per site, was recorded.

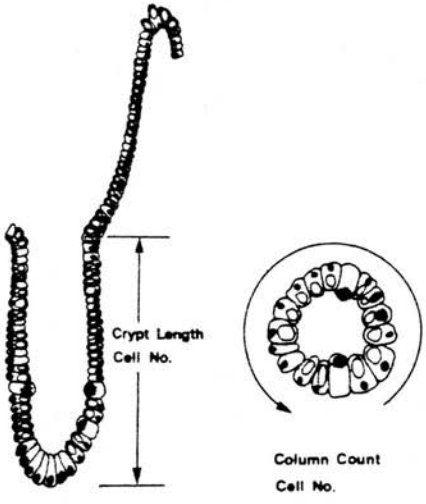

Figure 32.2 Enumeration of (a) number of cells per crypt profile, (b) number of cell per crypt column

During mitosis the cell protrudes into the lumen, consequently there is a greater chance of its appearing in a thin vertical section of the cylindrical crypt than if it remained in its normal position. Because this tends to give a misleadingly high number of mitoses per crypt, a correction, called 'Tannock's factor' must be applied[31,32]. A mitotic figure in a crypt cross-section

is chosen and using an eyepiece graticule the measurements necessary to make the following calculation are recorded (Figure 32.3):

$$\text{Tannock's factor} = \frac{\text{Distance from centre of crypt to centre of metaphase}}{\text{Distance from centre of crypt to centre of interphase cell nucleus}}$$

This was again calculated for 200 crypts per site.

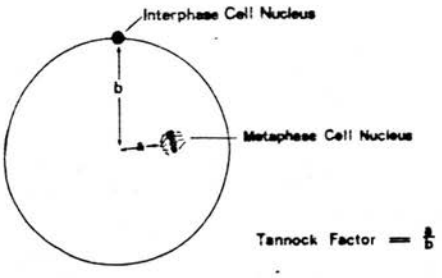

Figure 32.3 Estimation of Tannock's factor

Analysis of results

An estimate of the total number of cells per crypt was found from the product of crypt length and column count. Though this probably overestimates the actual number of cells, it provides a useful approximation of crypt size. The cell birth rate was estimated from the stathmokinetic data for the whole crypt, assuming a rectangular age distribution. The crypt cell production rate was derived by calculating the cell migration rate at the top of the crypt column from the stathmokinetic data for each cell position in the crypt, assuming an exponential age distribution at each position, and multiplying the migration rate by the column count. The relative proliferative zone size is the term we use here for the ratio to the crypt length of the cell position where the mitotic index falls to half its maximum value; the half-maximum position was estimated from the mitotic index distributions 120, 135 and 150 min after vincristine. It is likely that in the small intestine this relative proliferative zone size is close to the proportion of crypt cells proliferating, but in parts of the colon there may be a substantial number of non-proliferating cells in the proliferative part of the crypt. An estimate of cell cycle time has been made, ignoring this consideration, from the ratio of the relative proliferative zone size to the cell birth rate[32].

RESULTS

The animals tolerated anaesthesia and surgery well, post-operative mortality was low (< 5%) and post-operative infection was never seen. Both

groups of animals recovered quickly from the operations and gained weight at approximately equal rates. Approximately 8% of the patch-bearing animals spontaneously shed their exteriorized patches during the post-operative period for reasons which were not apparent.

Examination of the fixed small bowel from the 70% resection animals revealed lengthening, thickening and dilatation of the remnant. Microscopically the tissue architecture was unchanged at all sites examined, apart from slight thickening of all layers of the gut (Figure 32.4). The large bowel

Figure 32.4 (a) Ileum distal to the anastomosis 6 weeks after 70% resection. Hypertrophy of all layers of the gut is evident when compared to (b) control ileum 6 weeks after operation. (H and E × 40)

showed no obvious macroscopic or microscopic changes. Healing of the anastomosis was excellent; often the line of resection could be identified only as a small ridge on the internal aspect of the small bowel. Microscopically the continuity of the mucosa was usually maintained (Figure 32.5), although occasionally superficial ulceration was seen. The exteriorized patch of bowel remained moist with mucinous secretions throughout the post-operative period. Microscopically the most striking feature was the pronounced crypt hyperplasia (Figure 32.6) with loss of identity of crypt–villus junctions. The proliferative compartment was expanded and mitotic figures were seen up to cell position 70 in the crypts. Many crypts

Figure 32.5 Vertical section through the anastomosis 6 weeks after operation. Mucosal continuity is preserved. (H and E × 818)

Figure 32.6 Vertical section through an exteriorized patch 6 weeks after operation. The crypts are hyperplastic and show cystic dilatation; note the position of mitotic figures (arrowed): crypt–villus junctions are obscured. The villus epithelial cells are cuboidal or flattened. Inflammation with polymorph infiltration is present in the upper part of the lamina propria. (H and E × 200)

showed cystic dilatation. Cells in the upper parts of the crypt–villus structures were cuboidal or flattened in appearance and an infiltration of polymorphs was observed in the upper part of the lamina propria. Occasionally full-thickness ulceration of the mucosa was seen in parts of the patch. In the subserosa, massive formation of granulation and fibrous tissue was seen. Sectioning through the vascular pedicle suggested that the vessels remained patent, but it is likely that the patch also received nutrition from new blood vessels. Areas of foreign-body reaction with giant cells

were seen around the sutures. Blocks taken across the skin-patch junction revealed a sharp transition from squamous to columnar epithelial cells.

Table 32.1 shows the changes in crypt size parameters in the 70% resection animals; the crypt length increased compared to the patch animals, the changes being greatest immediately distal to the anastomosis; in small bowel the column count was also increased, but showed a reduction in the colon.

Table 32.1 Crypt size parameters

	Jejunum		*Proximal ileum*		*Distal ileum*		*Proximal colon*		*Distal colon*	
	70%	*Patch*	*70%*	*Patch*	*70%*	*Patch*	*70%*	*Patch*	*70%*	*Patch*
Crypt length (cells)	38.2	32.6	37.4	30.0	36.8	30.6	24.2	21.8	33.9	32.2
Column count (cells)	21.6	19.3	25.9	18.7	21.5	19.9	15.5	17.8	18.4	22.6
Cells/crypt	825	630	970	560	790	610	373	390	625	730

Table 32.2 shows the basic cytokinetic parameters for both operations, cell birth rate is reduced distal to the line of resection, crypt cell production rate is increased proximal and distal to the anastomosis and reduced at subsequent sites. Estimates of relative proliferative zone size are reduced and those of 'cell cycle times' increased distal to the line of resection.

Taken individually, most of the changes in the 70% resection group when compared to values for corresponding sites in the patch group are not statistically significant. When the overall trends are analysed these reveal a significant pattern of changes throughout the bowel. Table 32.3 summarizes the percentage increase in the values for the 70% resection animals when compared to the same values in the patch animals. It can be clearly seen that changes in crypt length, column count, total number of cells per crypt, cell birth rate and crypt cell production rate all show a negative gradient distal to the line of resection, and that changes in relative proliferative zone size and 'cell cycle time' show a positive gradient distal to the anastomosis.

Analysis of the exteriorized patch was rendered difficult by the loss of identity of crypt–villus junctions which made it impossible to estimate crypt length accurately. Since mitotic figures were observed up to cell position 70 in the crypt this was taken as the minimum crypt length. Table 32.4 summarizes the results obtained for the patch using three estimates of crypt length, 70 cells, 80 cells and 90 cells per crypt, and compared to those for the proximal ileum. Even using the most conservative estimate of crypt length the total number of cells per crypt is greatly increased, the cell birth rate is significantly lowered, but the crypt cell production rate is elevated, reflecting the increased number of proliferating cells in each crypt. The estimate of relative proliferative zone size is reduced and that of the 'cell cycle time' increased.

DISCUSSION

The results from the 70% resection animals confirm previous observations

Table 32.2 Cytokinetic parameters; cell birth rate, crypt cell production rate (CCPR), relative proliferative zone size and 'cell cycle time'

	Jejunum		*Proximal ileum*		*Distal ileum*		*Proximal colon*		*Distal colon*	
	70%	*Patch*	*70%*	*Patch*	*70%*	*Patch*	*70%*	*Patch*	*70%*	*Patch*
Cell birth rate (cells 1000^{-1} h^{-1})	38.0	30.7	28.6	38.4	22.0	33.3	4.3	8.2	5.2	10.6
CCPR (cells/h)	28.0	18.7	26.9	19.5	16.8	18.2	1.5	3.2	3.1	7.6
Relative proliferative zone size	0.65	0.74	0.62	0.73	0.71	0.72	0.71	0.64	0.67	0.59
'Cell cycle time' (h)	17.2	24.1	23.3	19.0	32.1	21.6	143	77	118	56

Table 32.3 Percentage changes in various parameters in the 70% resection animals compared with the values obtained for the same sites in patch animals, to illustrate the overall trends throughout the bowel

Site	*Jejunum*	*Proximal ileum*	*Distal ileum*	*Proximal colon*	*Distal colon*
Crypt length	18	25	20	11	5
Column count	12	39	8	-13	-19
Cells/crypt	31	73	30	- 4	-14
Cell birth rate	24	-26	-34	-48	-51
CCPR	50	38	- 8	-53	-59
Relative proliferative zone size	-12	-15	- 2	11	14
'Cell cycle time'	-29	23	49	86	111

Table 32.4 Results for the exteriorized patch of small intestine. Because the crypt-villus junctions could not be identified the crypt length could not be evaluated. Figures are given for three estimates of crypt length. Mitotic figures were not observed above cell position 70

Site	*Exteriorized patch*				*Proximal ileum*
Crypt length (cells)	*n*	*n* = 70	*n* = 80	*n* = 90	30.6
Column count (cells)	23.0	23.0	23.0	23.0	19.9
Cells/crypt	23*n*	1610	1840	2070	610
Cell birth rate (cells 1000^{-1} h^{-1})	1496/*n*	21.4	18.7	16.6	33.3
CCPR (cells/h)	32.7	32.7	32.7	32.7	18.2
Relative proliferative zone size	42/*n*	0.60	0.52	0.47	0.72
'Cell cycle time' (h)	28.1	28.1	28.1	28.1	21.6

that there is lengthening, thickening and dilatation of the remaining gut, this being particularly noticeable immediately distal to the anastomosis. Thickening of all layers of the bowel together with crypt hyperplasia is also confirmed. The crypt hyperplasia is reflected in the increased number of cells in each crypt profile and in the circumference of the crypt in some sites. The magnitude of these changes varies throughout the gut, with a negative gradient in the percentage change of crypt size parameters distal to the line of resection. These gradual changes have been observed by others[1], and strongly suggest a gradient of trophic stimuli down the gut. This seems to support the concept that the increased concentrations of luminal factors are the most important stimuli to adaptation of this type.

The cytokinetic parameters also show graded patterns of change. Use of the stathmokinetic method allows the calculation of cell birth rate and crypt cell production rate, the latter is considered to be of particular value in the assessment of kinetic changes in intestinal adaptation[33]. These parameters

also show a negative gradient of change distal to the anastomosis, again suggesting that the concentration of factors promoting the change decreases distally. The stathmokinetic method does not, however, allow accurate determinations or even comparisons of cell cycle times since these depend on knowing the proportion of proliferating cells, a quantity not readily estimated without using autoradiographical techniques; this could explain why our results, showing decreases in the size of the proliferative compartment and increases in cell cycle times, are in direct contradiction to previous work[3,4,7-9]. These parameters will be more accurately determined by tritiated thymidine labelling and fractions of labelled mitoses being currently studied.

Proximal to the line of resection there are also changes in crypt size and cytokinetic parameters, and whereas shortening of the gut brings abnormal concentrations of luminal factors into the distal remnant, this should not affect the proximal small bowel. It is possible that reflux into or prolonged transit times through this section of the bowel may occur, thus increasing the concentration of trophic factors, though in fact normal or reduced transit times have been reported by others[34]. Alternatively the changes could be brought about by humoral stimuli whose effects on the rest of the gut are masked by the greater effects of luminal factors.

Exteriorization of a patch of small bowel causes profound changes in crypt-villus architecture, crypt size and cytokinetic parameters, but these changes are restricted to the patch alone despite an apparently intact blood supply. These changes appear to be mainly attributable to traumatic effects on the surface of the mucosa: in a similar operation in the dog it has been reported that covering the explant with a skin flap results in reversion to an almost normal histological appearance[35]. This shows the bowel will respond with a hyperplastic reaction to a topical stimulus, in this instance mechanical trauma. The entirely local nature of this reaction suggests that only local control mechanisms are being invoked, though it should be noted that there is an intact autonomic supply via the pedicle. Evidence for the existence of autonomous local control systems governing crypt architecture and crypt cell production, migration and differentiation have come from studies of defunctioned gut[36] and of ischaemic villous damage[37] *in vivo* and also from the behaviour of intestinal mucosa in short-term organ culture[38]. Further evidence for this hypothesis has come from the recent observation that human colonic mucosa can be maintained in long-term organ culture[39] and is even capable of crypt regeneration after an initial degeneration phase *in vitro*[40].

The results of this study support the concept of epithelial cell production being governed by inherent local control mechanisms which are modulated by exogenous and endogenous factors according to the needs of the organism. The control of adaptation following proximal resection or exteriorization of a patch of bowel appears to be predominantly influenced by factors acting at the luminal surface.

Acknowledgments

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. Mr. C. J. Pritchett is in receipt of a Prophit Scholarship from the Royal College of Surgeons. We are grateful to Mrs E. Wallace and Mrs S. Robinson for technical assistance, to Mr S. Brabazon and Mr W. Robinson for producing the illustrations, and to Miss E. Wark and Miss C. Baker for typing the manuscript.

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Cell Tissue Kinet. (1986) **19**, 491–502.

A cytokinetic study of small-intestinal and colonic mucosa after resection of 70% of the small intestine

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(*Received* 28 *December* 1984; *revision accepted* 12 *April* 1986)

Abstract. Pulse-labelling with tritiated thymidine and a fraction of labelled mitoses experiments have been performed in order to investigate the proliferative changes induced at various sites in the hyperplastic small-intestinal mucosa of rats previously subjected to resection of 70% of the small intestine. Proliferative activity in the colon was also studied.

In the distal ileum there is a significant reduction in cell cycle time (T_c) of cells at all levels within the crypt and the growth fraction falls. In the jejunum and proximal ileum the crypts contain an increased number of proliferating cells, but as the size of the maturation zone is also increased, there is no significant alteration in the relative number of proliferating cells per crypt. Nor does the distribution of proliferating cells in these crypts seem to alter. There is no general reduction in T_c at these sites, but there does appear to be a significant reduction in T_c on the part of the cells in the stem-cell zone at the crypt base.

In neither proximal nor distal colon was there any significant proliferative change apparent after small-intestinal resection.

One approach which elucidates how epithelial cell renewal in the intestine is controlled has been to study the adaptive response of the remaining intestine following the surgical resection of a large part of the small bowel. It is now clear that, following major small-intestinal resections, the remaining segment of small bowel becomes thickened and elongated, and that there is hyperplasia of the mucosal crypts and enlargement of the villi; the changes are more marked distal to the resected segment than in the portion of bowel proximal to it (Booth *et al.*, 1959; Dowling & Booth, 1967; Nygaard, 1967). Their severity can be related to the proportion of the small intestine which has been removed (Hanson, Osborne & Sharp, 1977a). The adaptive response appears to begin within two days of resection (Obertop *et al.*, 1977) and to reach a new steady-state within two weeks or so (Hanson, Osborne & Sharp, 1977b). The altered state of cell proliferation persists for many weeks (Weser & Tawil, 1976; Williamson, Bauer & Malt, 1977) and therefore represents what presumably amounts to a stable adaptive response.

In the colonic mucosa there is also a brisk hyperproliferative response to small-intestinal

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resection which occurs within 24–48 hr of operation (Tilson & Michaud, 1976; Williamson, Bauer & Ross, 1978) and clear evidence of mucosal hyperplasia and increased cell proliferation has been documented at 14 days after resection (Nundy *et al.*, 1977). But while some suggestion of increased cell renewal has been shown several months after small-intestinal resection (Zufarov & Babekov, 1971), convincing evidence of sustained hyperplasia of colonic crypts is lacking (Williamson *et al.*, 1977; Senior *et al.*, 1982).

There are already a number of detailed studies which deal with several aspects of the long-term proliferative changes that occur in the intestinal mucosa as a result of small-intestinal resection (see Discussion), and the present authors have previously published observations that relate to crypt morphometry and crypt-cell birth rate in the small intestine and colon after resection (Senior *et al.*, 1982). In the present study we have used pulse-labelling with tritiated thymidine ([^{3}H]TdR) and the fraction of labelled mitoses (FLM) technique in order to expand our previous observations, and to document the nature of the proliferative changes that occur in cells at various levels within the crypt.

MATERIALS AND METHODS

Female Wistar Porton rats were used throughout. They were obtained from Olac Ltd., Bicester, and at the time of surgery were aged 21–23 weeks and weighed 170–210 g. They were fed on standard pellets (No. 3 Breeders' Diet expanded, SDS, Witham) and allowed water *ad libitum*.

Surgical procedures

Anaesthesia was induced by ether inhalation followed by 0·2 ml/kg body weight of pentobarbitone sodium solution ('Sagital', May & Baker, Dagenham, Essex) administered intraperitoneally (i.p.), and maintained by ether inhalation during surgery. The abdomen was opened by a 30–40 mm long midline incision and the small intestine exteriorized. Groups of animals were then subjected to one or other of the following surgical procedures.

Resection of 70% of the small bowel with jejuno–ileal anastomosis ("*Resection*")

A mesenteric vascular arcade 100–120 mm proximal to the ileo–caecal junction was selected and the mesentery split between vessels with iris scissors. An arcade immediately distal to the ligament of Treitz was similarly treated. The mesenteric vessels between these two points were ligated with 4/0 silk (Ethicon, Edinburgh) and the bowel divided proximally and distally. The intervening segment of bowel, which constituted some 70% of the total small intestine, was removed and intestinal continuity restored by end-to-end anastomosis with a continuous single layer of 8/0 silk (Ethicon). The intestine was replaced and 2–3 ml of sterile physiological saline introduced into the peritoneal cavity to replace fluid lost during surgery. The abdomen was closed with interrupted 4/0 silk sutures.

Exteriorization of a segment of ileum with ileo–ileal anastomosis ("*Patch*")

A branch of the superior mesenteric artery which supplies the ileum 100–120 mm proximal to the ileo–caecal junction was selected and the bowel on either side of it divided to produce a mobilized segment of ileum some 10–20 mm long on a vascular pedicle. The isolated segment of bowel constituted 5% or less of the total length of the small bowel, and intestinal continuity was restored by end-to-end anastomosis as above. The abdominal incision was closed around the pedicle of the mobilized segment which was then opened along its antimesenteric border and cleaned by flushing with physiological saline. An area of skin of

the approximate size and shape of the exteriorized segment was removed from the area around the pedicle and the segment sutured into place, mucosal surface outermost, with 6/0 silk. This operation comprises, for the purpose of this report, a control operation for 70% resection (see Discussion).

Food, but not water, was withheld for 24 hr post-operatively from both groups. After this time food was re-introduced. Both groups of animals recovered rapidly from the effects of their operations and resumed a normal level of activity and weight gain. Seven weeks after their operations the animals were used for the cytokinetic experiments described below. These experiments were commenced at 09.00 hours.

Cytokinetic studies

Labelling index study

Three animals from each group were given a dose of 1 mCi/kg body weight of [^{3}H]TdR (supplied by Amersham International, Amersham), by i.p. injection and killed 1 hr later. The specific activity of the [^{3}H]TdR was 5 mCi/mmol.

FLM study

At 09.00 hours, 23 animals from the 70% resection group and 22 animals with the transplanted patches were given a single injection of [^{3}H]TdR as above. Single resection animals were killed after 2, 3 and 8 hr and then at hourly intervals up to 18 hr; thereafter animals were killed at 2-hourly intervals up to 37 hr. Single patch animals were killed at 2, 3, 5 and 7 hr then hourly up to 18 hr and 2-hourly until 32 hr.

All animals were killed by ether overdose and subjected immediately to *post mortem* examination. The entire gut was removed and fixed, the small intestine being fixed unopened and the large intestine being first opened along its length and emptied. The exteriorized mucosal patches were fixed intact. Initial fixation involved immersion in Carnoy's fluid for 12–18 hr; thereafter the tissues were stored in 2-ethoxyethanol (Cellosolve). Transverse blocks for microscopy were obtained from the following sites in both groups of animals: (a) jejunum, 20 mm from the pylorus and thus proximal to the resection; (b) proximal ileum, 80 mm proximal to the ileo–caecal junction (and therefore distal to the resection site); (c) distal ileum, 30 mm proximal to the ileo–caecal junction; (d) proximal colon, 90% of the distance from the anal margin to the colo–caecal junction; (e) distal colon, 30% of the distance from the anal margin to the colo–caecal junction. Blocks were also obtained longitudinally from the site of the small-intestinal anastomosis and transversely across the exteriorized patches.

The blocks were processed routinely and embedded in Polywax (Difco, West Molesey), and 3-μm vertical sections cut. The sections taken from across the anastomoses and the skin-patch junction were stained with haematoxylin and eosin. The transverse sections, a–e, were submitted to autoradiography using Kodak AR 10 stripping film (Eastman Kodak, Rochester, New York) and a 4-week exposure period and then stained by Harris's haematoxylin. The slides from these sites were then randomized to avoid observer bias during the counting procedures.

Labelling index

The left sides of 100 perfect axial crypt sections were analysed at each of the sites a–e in the six animals which constituted the labelling index (LI) group. The total length of each crypt (in cells) was recorded along with the cell positions of labelled nuclei, position 1 being the cell

at the centre of the crypt base. Background labelling was very low in this material and the criterion for a labelled nucleus was taken as five or more grains lying directly above it. Whole-crypt labelling indices were obtained by dividing the actual number of labelled cells counted at a particular site in either group of animals by the total number of cells counted at that site. Labelling indices at each cell position within the crypt were calculated using the normalization procedure of Cairnie & Bentley (1967) as modified by Wright, Morley & Appleton (1972), using a standard crypt length; for ease of comparability this was the mean for that experimental group and for animals used previously in similar experiments (Senior *et al.*, 1982). The half maximum position (HMP), i.e. the cell position in the crypt at which LI falls to half its maximum value, was estimated. Since this is expressed as a proportion of crypt length, the actual standard crypt length used is unimportant.

FLM study

For the collection of the data for the FLM experiment, the crypts in the small bowel and the distal colon were divided into cell position groups each consisting of four cell positions numbered from the most basal cell. At least twenty mitoses were analysed for each group and the proportion labelled was ascertained. The mean value of the several cell position groups in a crypt was also obtained to give a 'whole-crypt' FLM. Because of the small number of mitoses and their restriction to the middle third of the crypt in the proximal large bowel, it was impracticable to collect cell position group data, and therefore the proportion labelled of at least eighty mitoses per whole-crypt was calculated at this site. For each site in the bowel whole-crypt FLM curves were constructed, along with FLM curves for individual cell position groups; the curves were fitted using the Gilbert program (Gilbert, 1972) which calculates T_c and the cell cycle phase durations and gives some indication of the variability of the cell cycle time.

A value for the growth fraction (I_p) of the whole-crypt was calculated by dividing the observed whole-crypt LI by a 'theoretical' LI which assumes all the crypt cells are cycling. This theoretical LI was obtained by dividing the estimates of the duration of the DNA synthetic phase (t_s) by the cell cycle time (T_c) as calculated from the FLM data. This I_p estimate assumes a rectangular age distribution.

The proportion of the crypt occupied by cycling cells was estimated by dividing HMP by crypt length: this gives an indication of the relative size of the proliferation zone within the crypt. An estimate of the growth fraction within this zone was obtained by dividing I_p for the whole crypt by the relative size of the proliferative zone.

RESULTS

The morphological and kinetic changes in the exteriorized patches of the patch animals are described elsewhere (Senior *et al.*, 1985). In the present communication, changes in the 70% resection animals will be compared with corresponding values for the appropriate sites in the intestine in continuity in the patch animals.

Morphological changes

Inspection of the small bowel from both groups showed that in the resection animals there was thickening and dilatation of the small bowel remnant when compared with that in the patch animals, and that there had also been some lengthening of the segment of the ileum distal to the site of the anastomosis. In neither group had the anastomosis itself caused any distortion of the gut visible to the naked eye. No obvious differences were apparent in the large intestine.

At the microscopical level the thickening of the wall of the small bowel in the resection animals was seen to have arisen as a result of both muscular hypertrophy and mucosal hyperplasia. Sections taken from across the anastomosis often showed a single or double ridge in the mucosa to mark the suture line and mucosal continuity was usually apparent, although in occasional animals from both experimental groups there were odd areas of indolent local mucosal ulceration. Neither inflammation nor ulceration was seen elsewhere in the small bowel of either group. No abnormalities were seen in the colon, and there was no obvious difference between resection and patch animals.

'Whole-crypt' kinetics

The estimates of 'whole-crypt' cell cycle phase durations and cell cycle times calculated from the analysis of the FLM experiment are presented in Table 1, and the fitted FLM curves are shown in Fig. 1 and 2. It can be seen that the general fit of the curves generated by the Gilbert program to the data points is good in most instances, but there are only a small number of data points defining the descending limb of the first peak which may reduce the accuracy of the estimates of t_s. The only significant alteration in cell cycle time is in the distal ileum where T_c has been reduced by resection. Labelling index distribution diagrams for the various intestinal sites in resection and patch animals are shown in Figs 3 and 4: in the two groups very similar patterns of labelling distribution are apparent in spite of the changes in crypt length at various sites. These latter changes, which we have documented elsewhere (Senior *et al.*, 1982) are shown in Table 2 along with estimates of other proliferative parameters dependent on crypt size and the [^{3}H]TdR labelling studies. The half maximum position (HMP), which is a measure of the absolute size of the proliferative compartment, is increased at all sites in the small bowel of the animals subjected to 70% resection; but the relative size of the proliferation zone in these hyperplastic crypts is unchanged or even slightly reduced due to a proportionate expansion in the size of the maturation zone. The estimates of growth fraction derived from the labelling studies are similar in the two groups, except for a significant reduction in the distal ileum of resected animals. The proportion of cells actually cycling within the proliferative zone is less than unity in both groups of animals and at all sites.

Table 1. Estimates of cell cycle time (T_c) and the durations of G_1, S and G_2 at different sites in the intestines of resection and patch animals.

	Site									
	(a) Jejunum		(b) Proximal ileum		(c) Distal ileum		(d) Proximal colon		(e) Distal colon	
	Resection	Patch	Resection	Patch	Resection	Patch	Resection	Patch	Resection	Patch
T_c (hr)	11·3	11·8	11·5	11·9	11·4*	17·8	32	29	46	56
	(0·2)	(0·3)	(0·2)	(0·4)	(0·3)	(0·8)	(1·1)	(0·8)	(6)	(6)
t_{G_1} (hr)	3·2	3·4	2·3	2·3	1·8*	7·5	20	18	35	45
t_S (hr)	6·7	6·9	7·7	8·0	8·0	8·7	10	9	8	8
t_{G_2} (hr)	1·5	1·5	1·6	1·5	1·6	1·6	2	2	3	3

Standard errors of the T_c are given in parenthesis. Statistically significant changes are marked with an asterisk. (For some discussion of the reliability of error estimates generated by the computer program see Al-Dewachi *et al.*, 1974.)

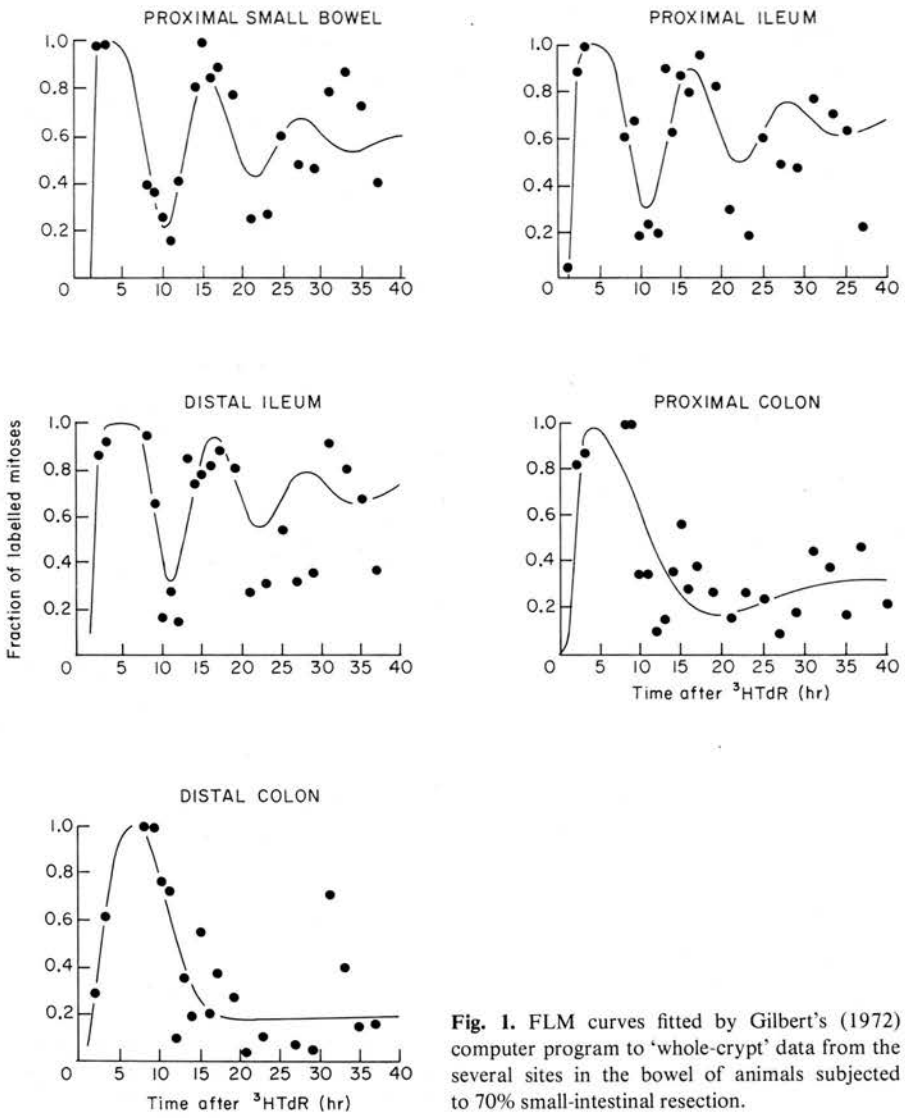

Fig. 1. FLM curves fitted by Gilbert's (1972) computer program to 'whole-crypt' data from the several sites in the bowel of animals subjected to 70% small-intestinal resection.

Kinetic changes at different levels in the crypt

Estimates of cell cycle time for cells at different levels within the crypts, at the four sites in the bowel where this type of analysis was practicable, are shown in Table 3. Considerable difficulty was experienced in obtaining a good fit to the experimental data using the Gilbert program, so the individual estimates are less reliable than those for the whole crypt, and this is particularly true of the distal colon. In the crypt base in the small intestine of the patch animals cell cycle times are considerably prolonged by comparison with cell positions higher up the crypt proliferation zone; this is a normal feature. In the resection animals, however, this feature is abolished at all three small-intestinal sites, T_c in the crypt base cells being much reduced. And while in jejunum and proximal ileum there is no obvious change in T_c in the major part of the proliferative zone after resection, a consistent reduction appears to have

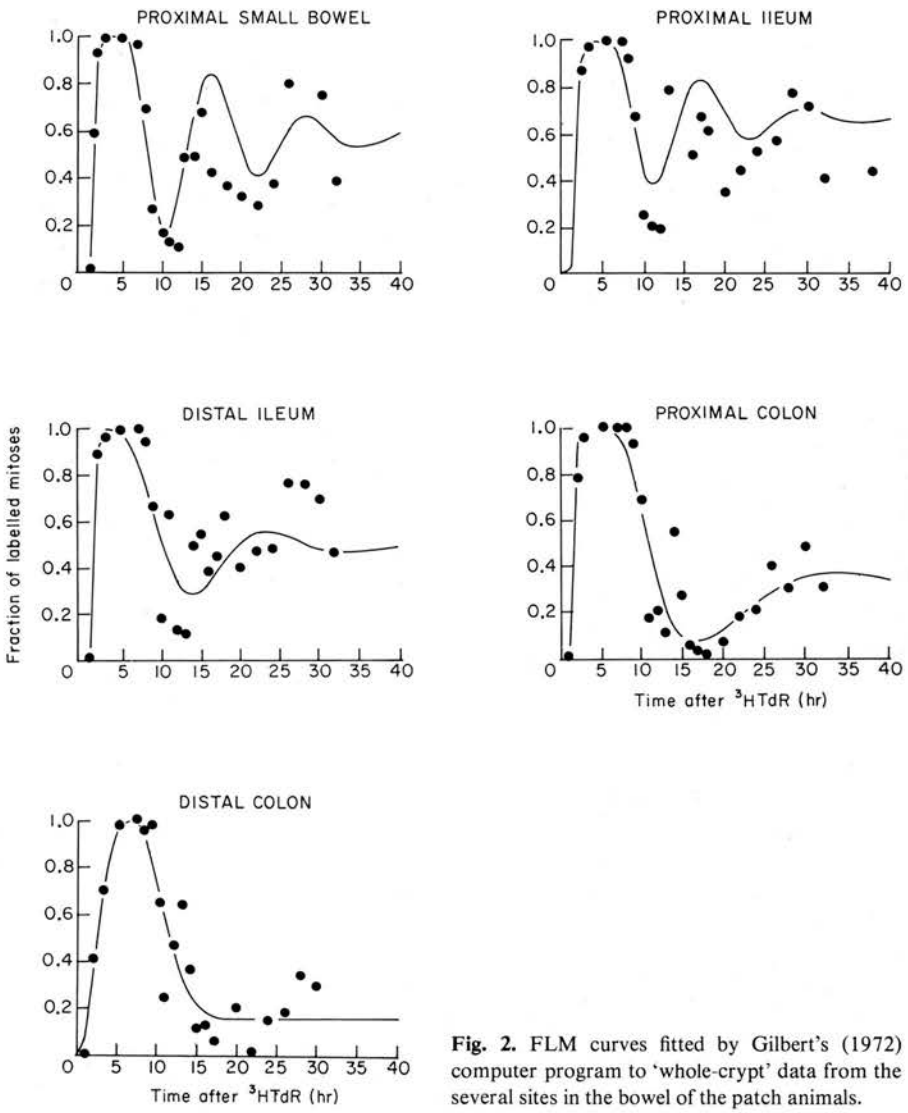

Fig. 2. FLM curves fitted by Gilbert's (1972) computer program to 'whole-crypt' data from the several sites in the bowel of the patch animals.

taken place in the distal ileum. In the distal colon, cell cycle times appear to be prolonged in resection animals when compared with patch animals, but considerable difficulty was experienced in fitting the FLM curves for the data from this site.

DISCUSSION

The aims of the present study were to examine the stable proliferative changes induced in small intestine and colon as a result of the resection some weeks previously of 70% of the small intestine. We have chosen to compare the results in our experimental group of animals with those from a cohort of animals kept at the same time and under identical conditions, and subjected to transplantation of a short segment of ileum to the anterior abdominal wall. These

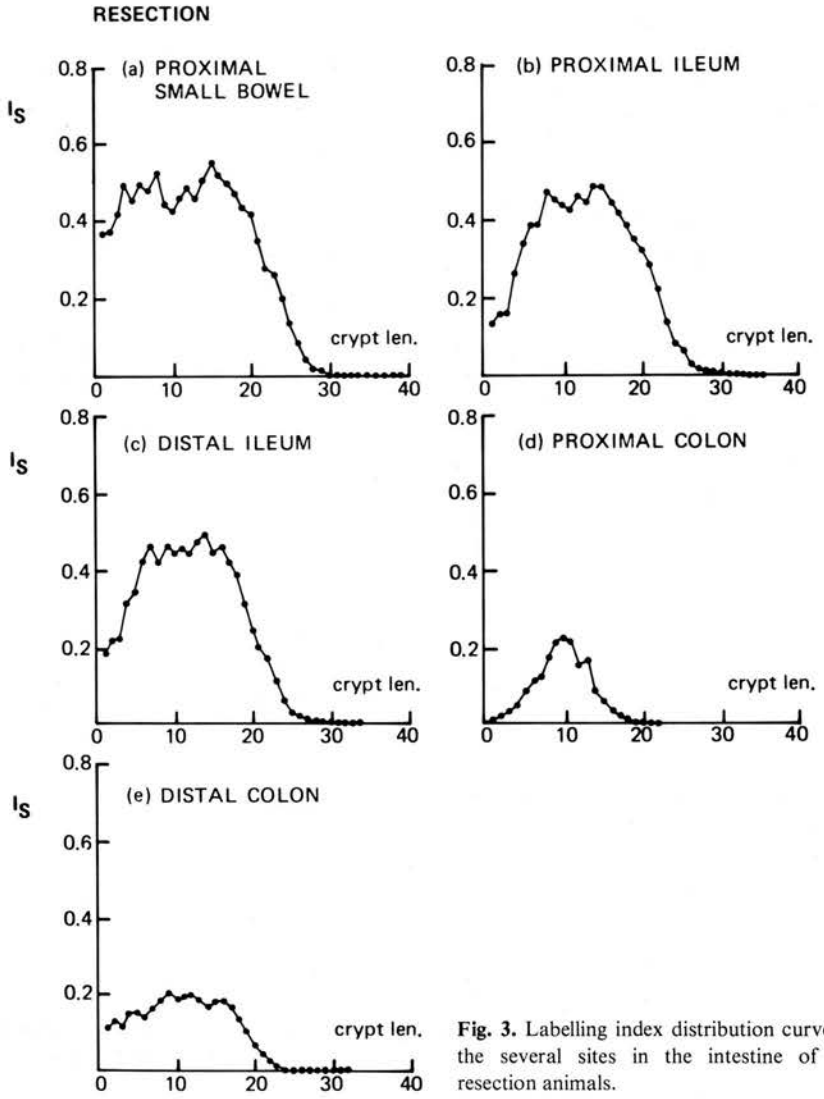

Fig. 3. Labelling index distribution curves for the several sites in the intestine of 70% resection animals.

animals therefore do not represent, strictly speaking, a control group, since such a group should be subjected only to division and re-anastomosis of the intestine. Given the results of Hanson and his co-workers (1977a), however, it seems most unlikely that the removal of less than 5% of the small intestine would have any measurable effect on cell proliferation in the rest of the bowel, and it is perhaps equally unlikely that the presence of an exteriorized segment would have any significant influence of the bowel in continuity. Bearing these factors in mind, we consider it is justifiable to use the patch animals as a standard by which to judge the effects of 70% resection.

What changes are then induced? As we and other workers have shown previously, crypt hyperplasia occurs in the residual small bowel. This hyperplasia has been shown to be associated with an increase in the amount of [^{3}H]TdR incorporated into the crypts as assessed by scintillation counting (Hanson *et al.*, 1977b) and an increase in the absolute

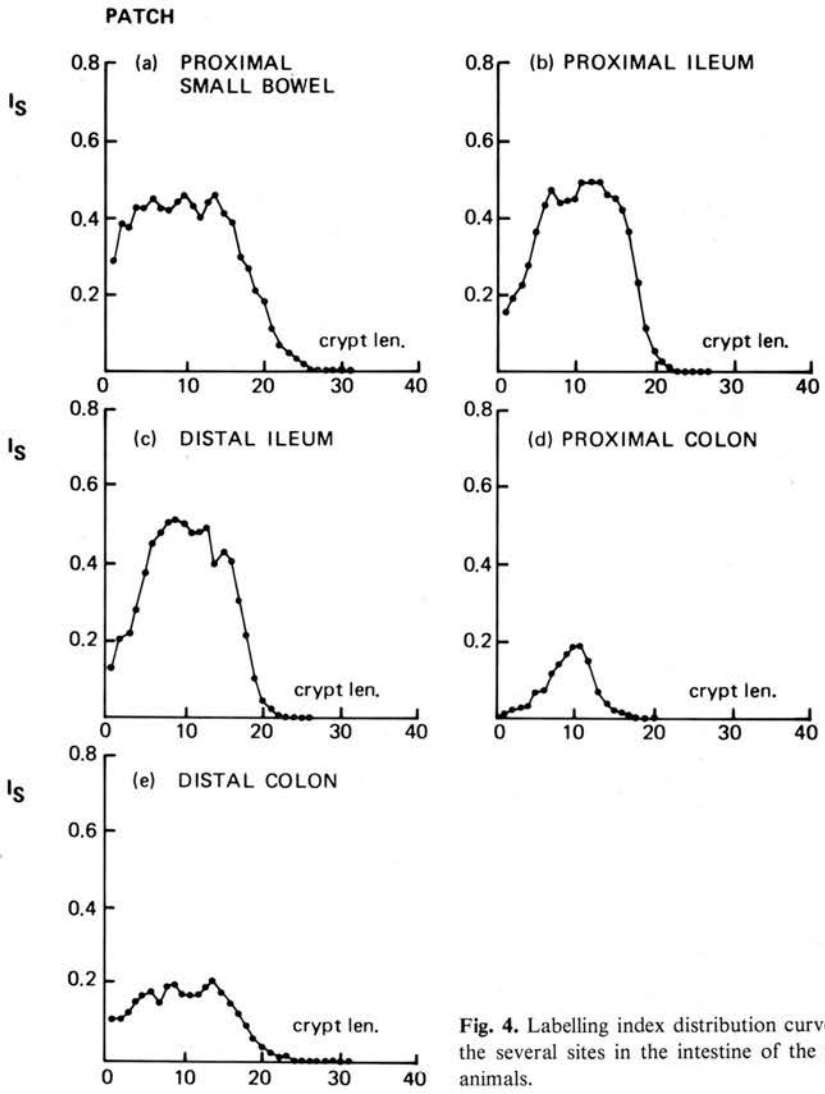

Fig. 4. Labelling index distribution curves for the several sites in the intestine of the patch animals.

numbers of DNA-synthesizing cells seen in the crypt (Hanson & Osborne 1971; McDermott & Roundnew, 1976; Hanson *et al.*, 1977a,b). Such changes are reflected in the present study by an increase in the absolute size of the proliferation zone as indicated by the increases seen in the HMP. The lack of any significant alteration in LI is largely explained by the fact that the relative size of the proliferation zone remains essentially unaltered after resection. Moreover, inspection of LI distributions (Figs 3 and 4) shows no significant alteration in the pattern of labelling within the enlarged crypts. As far as the large bowel is concerned, the present study has shown no significant change in HMP or in the relative size of the proliferation zone and no alteration in the pattern of distribution of labelled cells in the crypts. After a very similar small-intestinal resection, Sharp, Hanson & Osborne (1983) did demonstrate an increase in tritium activity in colonic crypts: their experiments were carried out at a shorter time post-resection however, and in view of the well-documented changes in

Table 2. Estimates of crypt length, half-maximum position (HMP), relative size of the proliferative zone (Pro), growth fraction (I_p), growth fraction within the proliferation zone (I_p(Pro)), and cell birth rate (k_b) at different sites in the intestines of resection and patch animals.

	Site									
	(a) Jejunum		(b) Proximal ileum		(c) Distal ileum		(d) Proximal colon		(e) Distal colon	
	Resection	Patch	Resection	Patch	Resection	Patch	Resection	Patch	Resection	Patch
Crypt length (cells)	38·2 (0·8)	32·6 (0·4)	37·4 (0·8)	30·0 (0·2)	36·8 (0·7)	30·6 (0·4)	24·2 (0·6)	21·8 (0·3)	33·9 (0·8)	32·2 (0·5)
HMP (cells)	22·5	18·5	22·0	18·0	21·0	17·5	14·0	13·0	19·0	17·5
Pro	0·57	0·59	0·60	0·60	0·58	0·67	0·65	0·65	0·60	0·56
I_p	0·46	0·42	0·36	0·36	0·35	0·51	0·22	0·18	0·36	0·39
I_p(Pro)	0·80	0·71	0·60	0·60	0·61	0·77	0·34	0·28	0·60	0·70
k_B (cells/1000/hr)	41	36	32	32	31	29	7	6	8	7

Standard errors for crypt length are indicated in parentheses. The S.E.M. of the HMP estimates is around 0·5 cells and for Pro 0·02–0·03.

Table 3. Estimates of cell cycle time (hr) and its coefficient of variation (%, in parentheses) at different cell positions at different sites in the intestines of resection and patch animals. Because many of the fitted FLM curves are poor fits, standard errors may be misleading and are not presented.

	Site							
	(a) Jejunum		(b) Proximal ileum		(c) Distal ileum		(d) Distal colon	
Cell positions	Resection	Patch	Resection	Patch	Resection	Patch	Resection	Patch
1–4	12·1 (14)	19·0 (28)	10·0 (58)	19·6 (23)	13·5 (24)	21·1 (25)	55·8 (59)	36·9 (87)
5–8	12·2 (17)	14·4 (23)	11·9 (18)	13·8 (23)	11·8 (17)	15·8 (26)	74·7 (83)	68·6 (94)
9–12	11·3 (17)	12·2 (15)	11·6 (16)	13·9 (27)	11·4 (14)	16·0 (36)	36·7 (80)	46·5 (78)
13–16	11·0 (16)	11·2 (11)	11·3 (16)	11·2 (13)	11·2 (15)	17·0 (29)	34·0 (77)	67·4 (99)
17–20	11·0 (16)	11·2 (11)	11·4 (15)	11·8 (15)	11·6 (12)	19·8 (31)	39·4 (57)	74·5 (47)
21–24	10·9 (16)	11·3 (14)	11·7 (15)	11·8 (15)	11·3 (12)	18·9 (29)	—	—

the colon which occurred immediately after resection (Tilson & Michaud, 1976; Nundy *et al.*, 1977), it is likely that the timing of the experiments is the cause of the apparent discrepancy between their results and ours.

Previous studies have shown only small reductions in cell cycle times in the small-intestinal crypts at various sites after major small-intestinal resection, and these have been associated with similarly unconvincing reductions in the duration of the DNA synthetic phase (Loran & Crocker, 1963; Hanson & Osborne, 1971; McDermott & Roundnew, 1976). In the present study we have confirmed that in the jejunum and proximal ileum no significant alteration in T_c occurs, but in the terminal ileum, while the growth fraction was reduced, T_c was shortened from 17·8 hr to 11·4 hr, due almost entirely to a shortening of the G_1 phase. Analysis of the FLM data by cell position group has also shown interesting results, but

because the data were difficult to fit, the conclusions should be treated with caution. In all three small-intestinal sites, resection induces a shortening of T_c in the cells at the crypt base; in jejunum and proximal ileum T_c is essentially unchanged elsewhere in the crypt, while in the terminal ileum T_c is reduced throughout the proliferation zone. The shortening of T_c at the crypt base may explain why previous workers (and indeed ourselves) have shown consistent, but very small, reductions in overall T_c assessed from 'whole crypt' data. More importantly, however, it represents a kinetic change that occurs locally in what has been termed the stem cell zone of the crypt (Cheng & Bjerknes, 1980). The number of stem cells per crypt remains in dispute: but one method of estimating it indirectly is the microcolony assay of Withers and Elkind, (1970). Using this technique, Hanson (1982) has shown that after intestinal resections crypt stem sensitivity to hydroxyurea is increased, implying a change in cell age distribution or a shortening of T_c, and that actual stem cell numbers, as estimated from colony survival after irradiation, may be increased. These observations are clearly explicable on the basis of the present finding of a significant reduction in T_c in the stem cell zone.

In the large intestine, significant changes in cell cycle time were not detected following resection. This is perhaps not surprising in view of the absence of any other significant crypt changes, but the evident variability would have made any changes difficult to detect.

The estimates of cell birth rate calculated from the LI data in the present experiment do not agree well with the values we have previously calculated from FLM data (Senior *et al.*, 1982). The reasons for this are not clear, but nevertheless the gradation of changes from proximal to distal were still apparent, and this implies the operation of luminal factors. Clearly, several kinetic changes occur within the crypts to effect an adequate adaptive response. The details of these changes vary from site to site, but the specific alteration of proliferation in the stem cell zone is likely to be of fundamental importance in the organisation of the response to resection.

ACKNOWLEDGMENTS

This work was supported by grants from the Northern Council of the Cancer Campaign. We would like to thank Mrs K. Elliot for technical assistance and Mrs A. Hogarth who typed the manuscript. Mr S. Brabazon prepared the illustrations.

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David Appleton

JOURNAL OF PATHOLOGY, VOL. **146**: 39–49 (1985)

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TRANSPLANTATION OF A SEGMENT OF ILEUM TO THE EXTERNAL ABDOMINAL WALL: AN ANIMAL MODEL OF INTESTINAL MUCOSAL HYPERPLASIA

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Received 1 June 1984
Accepted 8 October 1984

SUMMARY

When a segment of small intestine is transplanted to the external abdominal wall in rats adaptive changes occur in the exposed mucosa. These probably represent an extreme example of a physiological response to one type of trophic influence—the effect of mechanical trauma. The nature of the changes has been studied at 7 weeks after externalization using simple morphometry and a number of cytokinetic techniques (thymidine labelling, vincristine-induced metaphase arrest and the fraction-of-labelled-mitoses method), and comparisons drawn with the normal ileum. The exteriorized mucosa showed marked villus atrophy and hyperplasia of the crypts to three times normal size as a result of increases both in crypt length and crypt circumference. Neither metaplastic nor dysplastic epithelial abnormalities were observed. Crypt-cell production rate doubled in the hyperplastic crypts due to an increase in the size of the proliferation zone within the crypt, and the distribution of proliferating cells within the crypt changed. But cell cycle times were prolonged and more maturing cells were retained in the hyperplastic crypts. The potential usefulness of this model, particularly in carcinogenicity studies is considered.

KEY WORDS—Intestine, mucosa, transplantation, hyperplasia, vincristine, thymidine.

INTRODUCTION

Epithelial cell proliferation in the gut is normally a process in which cell production is balanced by

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0022–3417/85/050039–11$01·10
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cell loss. Perturbations of the proliferative system, whether directly by local trauma or the application of toxic agents, or indirectly by means such as the administration of trophic hormones or starvation, effect a rapid adaptive response on the part of the mucosa and result in the establishment of a new steady state of cell proliferation. This may be manifest morphologically as mucosal hyperplasia or atrophy. Clearly, then, sophisticated control mechanisms govern intestinal cell proliferation, and one approach to their elucidation has been to study adaptive responses to surgical manipulations of the bowel. A number of these studies have served to emphasize the importance of local trophic influences acting from the bowel lumen; for example, by-pass of a segment of small intestine results in atrophy of its mucosa,[1] whereas construction of blind but self-filling loops is associated with mucosal hyperplasia.[2] Simple resection of segments of bowel, with restoration of intestinal continuity, results in hyperplasia of the remaining bowel,[3] but in this situation local luminal influences may not be of overriding importance and recent attention has been focused on the possible role of systemic humoral factors in the intestinal adaptive response.[4] The complex subject of intestinal adaptation and its mechanisms has been the subject of a number of recent reviews,[5–8] and the possible relationship of adaptive hyperplasia to enhancement of carcinogenesis has received special attention.[9,10]

Whatever the fundamental controlling influences are, however, it is clear that as far as proliferating cells in the intestinal crypts are concerned only a limited number of alterations in proliferative behaviour are possible.[11] And it is also clear that local growth control mechanisms at the level of the crypt itself are highly sophisticated since mucosal growth and differentiation are well maintained even in long-term organ culture.[12,13]

The present study was designed to investigate morphological and cytokinetic changes in small-intestinal mucosa rendered hyperplastic as a result of the effects of local mechanical damage by virtue of its having been transplanted to the skin surface. In rats a small segment of ileum was isolated on a vascular pedicle and sutured, mucosal surface outermost, to the external abdominal wall: this results in mucosal hyperplasia restricted to the exteriorized segment.[14] Simple morphometric techniques involving cell counting procedures were used to compare the sizes of the crypts in the hyperplastic mucosa of the exteriorized ileum with the normal ileum in continuity and a number of cell kinetic techniques were used to define the nature of the changes responsible for the adaptive response. These techniques included stathmokinesis (metaphase arrest) with vincristine, from which the cell birth rate was calculated, flash labelling with tritiated thymidine, from which the proportion of cells in DNA synthesis was determined, and the fraction of labelled mitoses (FLM) method, from which the durations of the various phases of the cell cycle were calculated.

MATERIALS AND METHODS

Virgin female albino Wistar Porton rats were used; the animals were obtained from Olac Ltd, Bicester and at the time of surgery were aged 20–23 weeks and weighed 170–210 g.

Surgical procedure

Anaesthesia was induced by ether inhalation followed by 0·2 ml/kg body weight of pentobarbitone sodium solution (Sagital, May and Baker, Dagenham, Essex) administered interperitoneally. Anaesthesia was maintained by ether inhalation during surgery. The abdomen was opened by a 30–40 mm long midline incision and the small intestine exposed.

A branch of the superior mesenteric artery supplying the ileum 100–120 mm proximal to the ileocaecal junction was chosen and the bowel on either side of it divided to produce a mobilized 10–20 mm long segment on a vascular pedicle. Intestinal continuity was restored by end-to-end anastomosis with a single continuous layer of 8/0 silk (Ethicon, Edinburgh) and the bowel replaced in the abdomen. The abdominal wall was closed around the pedicle of the mobilized segment of ileum with interrupted 4/0 silk (Ethicon), care being taken to preserve the integrity of its blood supply. It was then opened along its antimesenteric border and cleaned by irrigation with sterile physiological saline. An area of skin of the approximate size and shape of the opened segment was removed from the area around the pedicle and the segment sutured into place, mucosal surface outermost, with 6/0 silk (Ethicon) to form a 'patch'.

Food, but not water, was withdrawn for 24 h post-operatively. After this time animals were allowed food (No. 3 Breeders Diet expanded, SDS Witham) and water *ad libitum*. The experiments described below were carried out seven weeks after

the operation. All experiments were commenced at 09.00 h.

Kinetic experiments

Stathmokinetic study—Nine animals were given a standard dose of 1 mg/kg body weight of vincristine sulphate (Oncovin, Eli Lilly, Basingstoke, Hants) by intraperitoneal injection. Thereafter they were killed serially at 15 min intervals commencing 30 min after injection.

Labelling index study—Three animals were given a dose of 1 mCi/kg body weight of tritiated thymidine (^{3}H-TdR) (Amersham International, Amersham) by intraperitoneal injection and killed 1 h later. The specific activity of the ^{3}H-TdR was 5 mCi/mmol.

FLM study—At 09.00 h 22 animals received a single injection of ^{3}H-TdR as above. Single animals were killed at 2, 3, 5 and 7 h then hourly until 18 h and then at 2 h intervals until 32 h.

All animals were killed by ether overdose. The entire gut from the pyloric end of the stomach to the anal fur was removed immediately *post mortem*, and the small bowel was fixed unopened. The exteriorized patches were removed and fixed intact. All tissue was placed for an initial 12–18 h in Carnoy's fluid after which it was transferred to 2-ethoxyethanol. For the kinetic studies a transverse block was taken from the ileum 80 mm proximal to the ileocaecal junction, i.e. close to but not at the site of origin of the patch and another through the central portion of the exteriorized patch. Additional blocks were taken longitudinally through the line of the intestinal anastomosis and across the skin-patch junction to assess healing.

The blocks were processed into Polywax (Difco, West Molesey) and 3 μm vertical sections cut. The sections from the vincristine-treated animals, and from all blocks taken from the site of anastomosis and from the skin-patch junction, were stained with haematoxylin and eosin. Slides from the ^{3}H-TdR treated animals were submitted to autoradiography using Kodax AR10 stripping film (Eastman Kodak, Rochester, New York) and a 4 week exposure period, and then stained by Harris's haematoxylin.

Collection and analysis of data

Stathmokinetic experiment—In the samples from the proximal ileum and in the transverse sections of the patches the left-hand sides of one hundred perfect axial sections of crypts were analysed. The cell position at the bottom of the crypt was taken as position one and the total length of each crypt was recorded in cell units, along with the cell positions of arrested metaphases. In the ileum continuity identification of the crypt-villus junction was easy, but this was not the case in the patches (see Results section). Cross-sections of crypts were obtained by turning some of the blocks, and for each site the circumferences of two hundred crypts were recorded in cells (crypt column count). On the same material Tannock's factor,[15] which is applied to compensate for overestimation of mitotic index due to the selection for counting of axial sections, was calculated.

An estimate of the total number of cells per crypt was made from the product of mean crypt length and mean column count. This is probably a slight overestimate but is a useful index for comparative purposes. The mitotic index data were projected on to a 'standard' crypt, the length of which was the mean at that site for that experimental group, using a modification[16] of the computer method of Cairnie and Bentley[17]. Cell birth rate for the whole crypt was estimated from regression analysis of whole crypt mitotic index versus time after vincristine and a rectangular age distribution was assumed for this analysis. Crypt cell production rate (CCPR) was calculated from the product of column count and cumulative birth rate at the mouth of the crypt. This latter value was obtained by summation of the birth rate estimates calculated for individual cell positions within the crypt. The mitotic index data were obtained from the distribution diagrams and the regression analyses performed assuming an exponential age distribution at these individual cell positions.[18]

Labelling and FLM experiments—The whole-crypt labelling indices were estimated by counting the number of labelled cells in the 'left hand' columns of 100 crypts and dividing by the total cell number. Labelling indices at each cell position in the standard crypt were calculated using the same normalization procedure as that described above for mitotic index data, and plotted against cell position. The half maximum position (HMP), i.e. that cell position in the crypt at which labelling index falls to half its maximum value, was estimated.

For the collection of the data for the FLM experiment the crypts were notionally divided into cell position groups each consisting of 4 cell positions numbering from the most basal cell. At least twenty

mitoses were analysed for each cell position group the proportion of them labelled being ascertained. For each set of observations a mean value was also calculated to give a 'whole-crypt' FLM. Graphs of the proportion of mitoses labelled against time were produced for individual cell position groups and for the whole crypt; these FLM curves were fitted using the Gilbert program[19] which calculates the cell cycle time (T_C) and the cell-cycle phase durations and gives some indication of the variability of the estimates.

A value of the growth fraction, that fraction of crypt cells actually in the proliferative cycle, was estimated by dividing the observed labelling index, obtained by actual cell counts, by a calculated value of labelling index which assumes all the cells are cycling. This 'theoretical' labelling index was arrived at by dividing the duration of the DNA-synthetic phase by the cell cycle time, as estimated from the FLM experiment. The proportional size of the proliferative compartment of the crypt was estimated by dividing the half-maximum-position (in cells) by the crypt length (in cells). An estimate of the proportion of cells in this zone actually in the proliferative cycle was made by dividing whole crypt growth fraction by the proportional size of the proliferative compartment.

RESULTS

The animals generally tolerated the anaesthetic well, but occasionally exhibited bronchospasm after a short exposure to ether. It was found that animals so affected could usually be resuscitated and surgery continued. The animals tolerated surgery well and post-operative mortality was low (less than 5 per cent), the usual cause of death being failure of the anastomosis. Within 6–8 h of the operation the animals had fully recovered from the effects of the anaesthetic and they began to eat as soon as food was made available. Ten per cent of the rats shed their exteriorized patches over the post-operative period; examination of such animals revealed perfect healing of the abdominal wound.

Gross examination and histological findings

Visual inspection of the small bowel prior to fixation showed no evidence that the anastomosis had caused any distortion of the gut, nor was there any suggestion of stricture formation. Sections taken across the anastomoses showed in most cases only a small single or double ridge on the luminal surface to mark the suture line. Mucosal continuity was usually seen, although in some cases local indolent ulceration was present.

The exteriorized patches remained moist with mucous secretions throughout the post-operative period. Microscopically there was marked crypt hyperplasia with mitotic activity evident up to cell position 70 (Fig. 1). Apart from this crypt architecture was essentially normal, although old areas of cystic dilatation of some crypts by mucus were seen in some patches. The crypt–villus junctions were obscured and the villus architecture was abnormal, there being subtotal villus atrophy. The villus remnants were covered by a cuboidal or flattened epithelium. An active inflammatory infiltration, with a prominent polymorph element was present in the upper parts of many villi demonstrating the effects of continuous trauma and this inflammation was especially noticeable in the occasional areas of superficial ulceration which were apparent. There was usually formation of granulation tissue in the deeper layers of the patches together with reparative fibrosis and neo-vascularization. This probably augmented their blood supply, but nevertheless sections through the pedicles showed that the original mesenteric vascular supply remained intact. A foreign body granulomatous response was conspicuous around the suture material. The junction between the epidermis of the abdominal skin and the columnar epithelium of the patch remained remarkably sharp with neither a transition zone nor any tissue defect (Fig. 2).

In none of the patches was there any suggestion of any dysplastic abnormality of the epithelium.

Morphometric and kinetic changes

The morphometric and kinetic results from the exteriorized patch, along with the corresponding values for the ileum in continuity are presented in Table I. Because the crypt–villus junctions were ill-defined in the patches the lengths of their crypts could not be evaluated accurately. Therefore, since mitotic figures were observable within crypt structures up to cell position 70, and, since despite the mucosal abnormalities, mitotic activity was never seen on villus remnants, an estimate of the mean crypt length of 80 cells has been made for the patches. This represents the minimum probable crypt length and will therefore yield conservative estimates for those values dependent on crypt length.

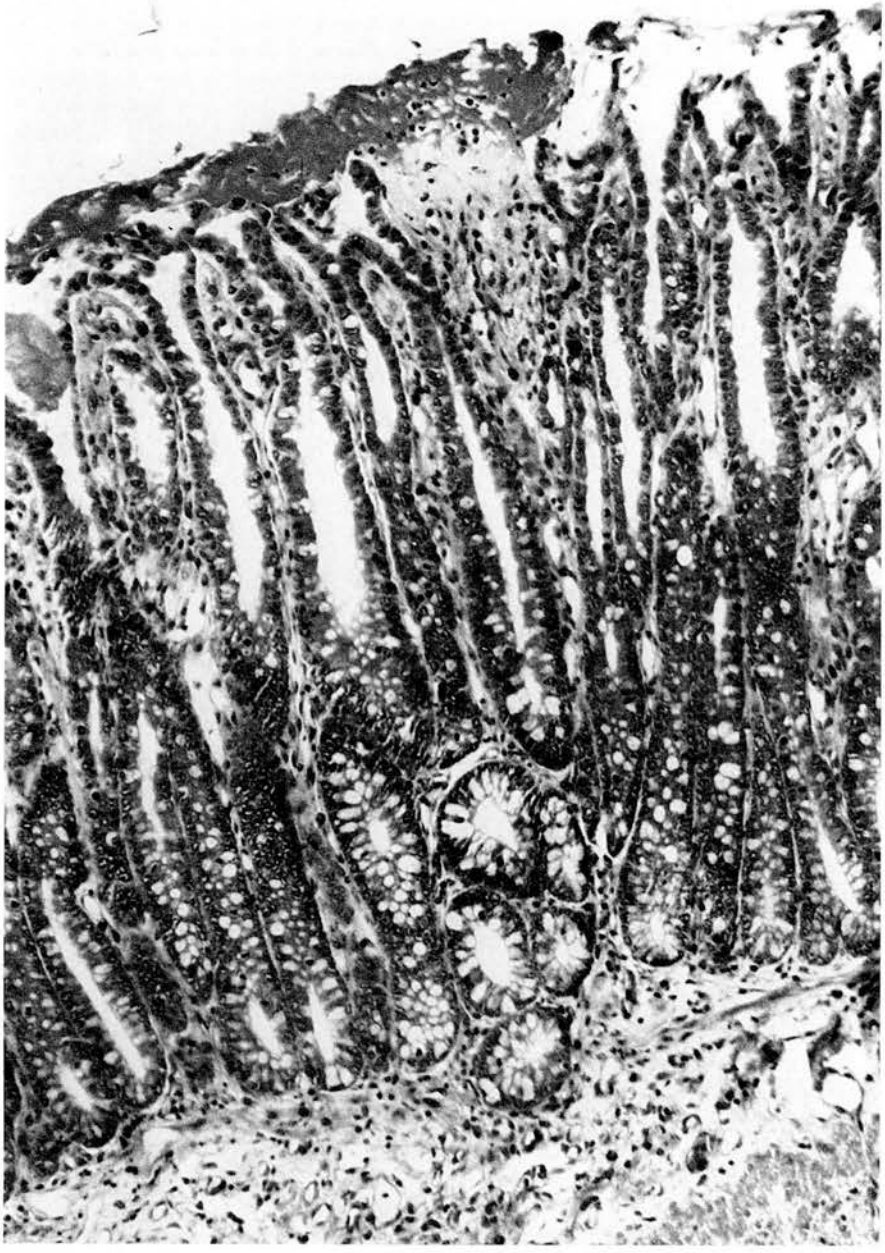

Fig. 1—A vertical section taken through the central portion of one of the exteriorized patches. Marked crypt hyperplasia is evident, and in this example some patchy surface erosion. H and E, × 225

Even the conservative estimate for crypt length in the patches is approaching three times the value for normal ileum. The crypt circumference too is increased in the patches and overall crypt cell population in the hyperplastic crypts of the patches is thus more than three times the corresponding value for the normal ileum. Calculation of the cell birth rate directly from the metaphase arrest (stathmokinetic) experiment gives values which are similar to the birth rate estimates obtained indirectly using cell cycle time and growth fraction data from the labelling studies. Both show a clear

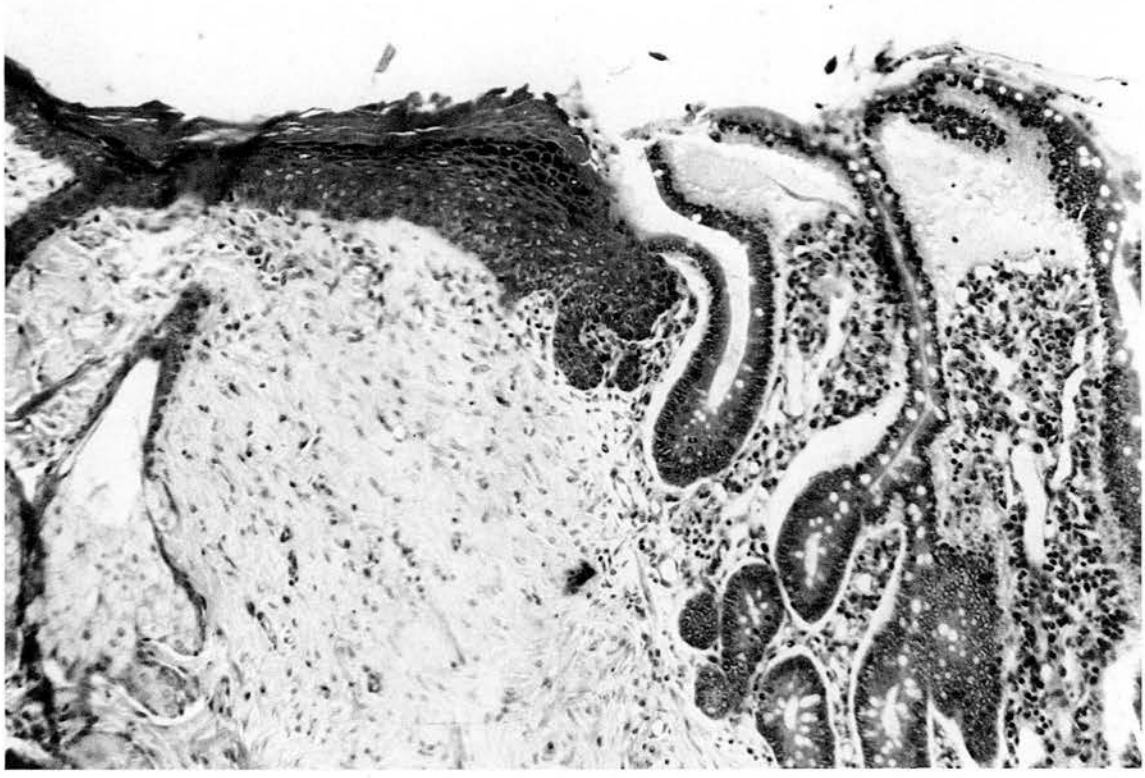

Fig. 2—The skin–patch junction at the edge of one of the exteriorized patches. The transition between cutaneous stratified squamous epithelium and mucous secreting mucosa is remarkably clear-cut. H and E × 203.

reduction in birth rate in the epithelium of the exteriorized patches. Calculation of the crypt cell production rates from the metaphase arrest date again show good correspondence with the values obtained from the labelling studies: the hyperplastic crypts of the patches are seen to be producing about twice as many cells each as are the normal ileal crypts. The cell cycle time, calculated from whole crypt fraction-of-labelled-mitoses data, is increased in the epithelium of the patch and inspection of the values for the cell cycle phase durations shows that this increase is due to prolongation of G_1 phase. The FLM curves fitted by Gilbert's[19] program are shown in Fig. 3; for the patch the second peak plateaus at about 0·5 suggesting variability in cell cycle time within the population mainly due to variability in the duration of G_1 phase. In the labelling index distribution curve of the patch (Fig. 4) the site at which labelling index falls to half its maximum (which gives an indication of the proportion of the crypt actually occupied by proliferating cells[20]) is increased from its normal level of cell position 18 to cell position 35; thus the absolute size of the proliferation zone in the hyperplastic crypts is increased. But relative to total crypt size the proportion of the crypt containing proliferating cells, calculated from the ratio of the half maximum position total crypt length, is reduced in the hyperplastic crypts. Inspection of the labelling index distribution diagram, however, reveals a long tail of low labelling indices up to cell position 60, suggesting that the half maximum position is less appropriate than usual as a summary of the upper limit of the proliferative compartment, and that we may therefore be underestimating the proportion of proliferating cells. This reservation is justified when the whole-crypt growth fraction is calculated from the labelling index data, since this is found to be similar in the hyperplastic and normal mucosae. The proportion of cycling cells within the proliferative zone is increased in the hyperplastic crypts but this figure may be an overestimate due to the uncertainty of what the extent of the proliferative compartment actually is.

The cell cycle times at the individual cell position groups within the crypt, up to cell position 28, are

Table I—Whole-crypt morphometric and kinetic parameters for the exteriorized patch based upon an estimate of 80 cells for crypt length

	Exteriorized patch	Ileum
Crypt length (cells)	80·0*	30·0(±0·7)
Crypt circumferences (cells)	23·0	18·7
Crypt population (cells)	1840*	560
Birth rate (calculated from vincristine data, cells/1000 cells/h)	18·7*	38·4(±7·9)
Birth rate (calculated from FLM data, cells/1000 cells/h)	20·0	30·2
Crypt/cell production rate (calculated from vincristine data, cells/h)	32·7	19·5
Crypt/cell production rate (calculated from FLM data, cells/h)	36·8	18·2
Cell cycle time (h)	15·6(±0·5)	11·9(±0·4)
Duration of G_1 phase (h)	6·7(±0·5)	2·3(±0·4)
Duration of S phase (h)	7·3(±0·1)	8·0(±0·3)
Duration of G_2 + M phase (h)	1·6(±0·1)	1·5(±0·1)
Half maximum position	35	18
Relative size of proliferation zone	0·44*	0·60
Labelling index	0·145*	0·242
Growth fraction	0·31*	0·36
Growth fraction on the proliferation zone	0·70	0·60

* Estimates dependent on the crypt length. Corresponding estimates are shown for the ileum in continuity.

The standard errors where available are given in parenthesis: the values derived from Gilbert's program[19] should be interpreted with caution.[31]

presented in Table II. The cell cycle times are prolonged in the basal region of the crypt due to a slight increase in the duration of the G_1 phase, but it should be noted that the FLM curves in this region proved very difficult to fit using the Gilbert program.

DISCUSSION

The principle changes in the mucosa of the exteriorized small-intestinal patches are crypt hyperplasia and marked villus atrophy, and it is easy to see how comparisons with the state of the human small intestine in coeliac disease have been drawn.[14] The normal differentiated cellular elements are retained in the crypts; the mucosal surface is not subjected to epithelialization by stratified squamous epithelium; and neither metaplastic nor dysplastic epithelial abnormalities are observed. These latter points are of importance in interpreting the significance of the underlying proliferative abnormalities in this model of mucosal hyperplasia, in view of the reported observation of spontaneous neoplastic change at the site of large-intestinal stomas in rats.[21] In the present study there was no morphological evidence of any stage of neoplastic development in the patches: and in the absence of metaplastic or dysplastic changes we feel confident that the model truly represents the response of normal small intestinal mucosa to an unusually severe trophic stimulus. In support of this view it has been shown in dogs that covering exteriorized mucosa results in a return to normal appearances.[22]

The cause of the trophic stimulus must be markedly increased loss of surface epithelial cells as a result of simple mechanical damage. Histologically there is clear evidence of continued trauma in the form of active surface inflammation and focal ulceration. While the cause of the increased villus-cell loss is not physiological in this case, there are certainly diseases where enhanced villus-cell loss is the major stimulus to compensatory hyperplasia, for example coeliac disease in man,[23] and the self-limiting nematode infestation in rodents due to *Nippostrongylus brasiliensis*[24].

The stimulus to cell proliferation provided by exteriorization of the intestine is very marked; the

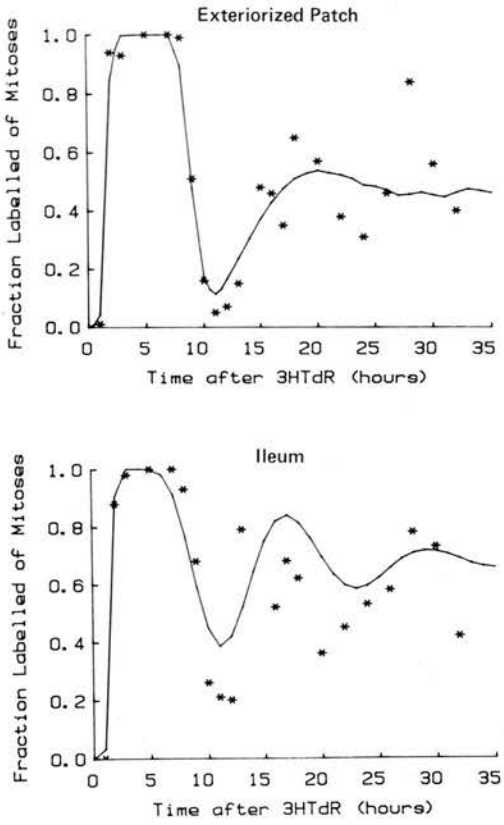

Fig. 3—FLM curves for the exteriorized patch and the normal ileum (whole crypt) fitted by the Gilbert program

hyperplastic crypts are around three times normal size. Not surprisingly this is a much more conspicuous change than that seen following the normal physiological demands of lactation,[25] or hypothermia-induced hyperphagia.[26] It is also a much more marked response than that seen after experimental subtotal intestinal resections[27] and after the administration of allegedly enterotrophic hormones.[28] Nor does the hyperplasia induced by chronic administration of the intestinal carcinogen dimethylhydrazine[29] approach in its severity the extent of the change seen in this model. It might be argued therefore that the ectopic transplantation model represents a completely unrealistic situation, where the intestine is subjected to a degree of injury far in excess of that ever encountered physiologically: but there are situations where natural diseases causing increased enterocyte loss are associated with a similar degree of crypt hyperplasia to that seen in the present model.[23] We are confident, therefore, that the model does provide a valid approach to the investigation of a physiological adaptive response.

We have chosen to compare the marked kinetic changes occurring in the exteriorized segments with the proliferative characteristics of the ileum in continuity in the same experimental animals. The blocks for comparison were selected from a site close to the donor site, but far enough removed from the anastomotic line to avoid local perturbations; and we feel this proximity is justifiable in view of the conspicuous absence of major surgical complications such as stricture, peritoneal fibrosis, or infection. It is not likely that the shortening of the bowel occurring as a result of removal of the patch will effect any change in proliferation in the bowel in continuity,[27] and the practical advantages of not using a formal control group outweigh any theoretical objections, given the difficulties of matching food intake etc. in different groups of animals.

The estimates of birth rates derived from the vincristine data correlate very well with the estimates arrived at as a result of the labelling studies and both experiments show that as far as the crypt as a whole is concerned birth rate is substantially reduced in the transplanted patches. This is probably partly the result of an increase in the numbers of non-proliferating cells retained in these hyperplastic crypts, i.e. at least part of the expansion of the crypts is caused by an increase in the numbers of maturing cells. A further factor which obviously contributes to the fall in crypt-cell birth rate in the patches is lengthening of the cell cycle time, due mainly to a lengthening of the G_1 phase of the cycle. Despite these changes, which militate against enhanced crypt cell production, the estimates of crypt cell production rate both show considerable increases in the patch. This is achieved as a result of the greatly increased size of the crypts, and in particular an increase in the size of the proliferation zone within the crypts. Overall the growth fraction in the crypts shows little alteration, but there is some suggestion of an increase in this parameter in the proliferation zone.

Analysis of the FLM data for the different parts of the crypts shows that in the normal ileum cells in the basal part of the crypt are cycling more slowly than those higher up the crypt, and this is a situation which is well documented in normal animals.[29-31] This is in accord with the concept of a slowly-cycling stem-cell zone.[32] In the patches cell cycle times are prolonged throughout the proliferation zone and the cell cycle time at the crypt base does not appear to be altered.

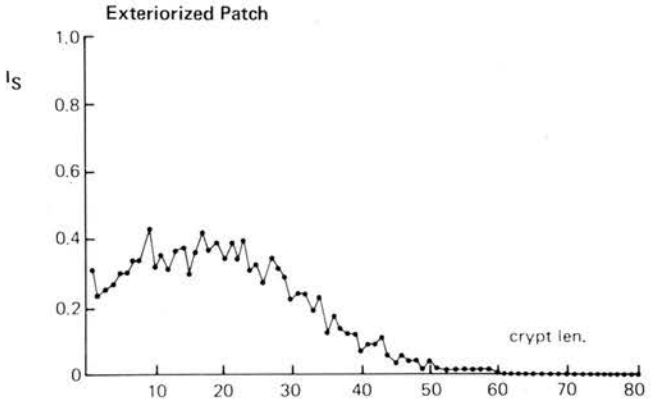

Fig. 4—Labelling index distribution diagram for the exteriorized patch

In summary therefore the present study has shown that chronic trauma produces massive crypt hyperplasia and doubles CCPR largely by increasing the absolute size of the proliferation zone. Cell cycle times in the proliferating cells are however prolonged, the normal distribution of proliferating cells is altered, and more maturing cells are retained in the crypts. There does not appear to be acceleration of proliferation in the stem-cell zone. The clear nature of these morphometric and kinetic changes suggest that this model represents an extreme example of one type of physiological response, and that it may thus be an ideal situation in which to study the dynamic response to topical trauma.

Further applications of the model may however be found in the study of intestinal carcinogenesis. As is the case in most internal viscera the sequences of morphological and proliferative changes which occur during intestinal carcinogenesis are at present incompletely understood, owing to their having to be implied from autopsy material. Exposed patches of intestine could be observed directly during the process of carcinogenesis due to systemic or topical application of carcinogens, and the sequence of changes monitored by inspection or even by biopsy. It is also possible that similar direct observations could be made on factors influencing the process of carcinogenesis. In relation to this latter point it is worth remembering that in a number of experimental situations it has been suggested that non-

Table II—Values for cell cycle time (h) and its coefficient of variation (C.V.) by cell position group for the exteriorized patch and for the ileum in continuity

	Exteriorized patch		Ileum	
Cell position	Cell cycle time (h)	Coefficient of variation (%)	Cell cycle time (h)	Coefficient of variation (%)
1–4	18·4 (±0·7)	45	19·6 (±0·6)	23
5–8	16·3 (±0·6)	38	13·8 (±0·4)	23
9–12	16·5 (±0·8)	40	13·9 (±0·8)	27
13–16	17·1 (±0·8)	42	11·2 (±0·2)	13
17–20	15·5 (±0·6)	24	11·8 (±0·4)	15
21–24	13·4 (±0·7)	19	11·8 (±0·3)	15
25–28	15·0 (±0·7)	22		
29+	15·2 (±0·6)	23		

The quoted standard errors of the cell cycle time estimates (given in parenthesis) should be treated with caution, as they are certainly underestimates, probably by a factor of two or three, of the real standard errors.[31]

specific intestinal mucosal hyperplasia can have a promoting effect on chemical carcinogenesis.[9,10] This hypothesis has been questioned however and the data are contradictory.[33] This is an area which may have direct relevance to man,[34] and there are therefore good reasons for investigating any possibility of promotion of carcinogenesis by intestinal mucosal hyperplasia. Experiments on animals bearing exteriorized patches may provide a means of determining whether such an effect does exist, and how it is mediated.

ACKNOWLEDGEMENTS

This work was supported by grants from the Northern Council of the Cancer Research Campaign. We would like to thank Mrs K. Elliott for technical assistance and Miss A. Lott who typed successive versions of the manuscript. Mr S. Brabazon prepared the illustrations.

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Cell Tissue Kinet. (1987), **20**, 43–56.

A trophic effect of epidermal growth factor (EGF) on rat colonic mucosa in organ culture

32

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(*Received* 10 *February* 1986; *revision accepted* 11 *October* 1986)

Abstract. The development of an organ-culture system for rat colonic mucosa has enabled a direct assessment of the effect of epidermal growth factor (EGF) on cell division. An augmented mitotic index (AI_m) has been employed to identify changes in cell proliferation.

Explants of colonic mucosa from four animals were maintained in a medium containing serum for five days. On the fifth day of culture, half of the explants received fresh medium containing EGF (40 ng/ml) and the remainder (controls) fresh medium only. At 6, 12, 24 and 48 hr thereafter groups of both experimental and control explants received the metaphase-arresting drug vincristine (4 μg/ml) for 3 hr prior to fixation. The proportions of vincristine-arrested metaphases within the explants were determined. Analysis of the data indicates that when serum is present exogenous EGF exerts a trophic effect which increases with time ($P < 0{\cdot}001$).

In a second experiment colonic explants from four animals were maintained for five days in a serum-free medium and were then divided into groups, each of which received one of a range of concentrations of EGF. The AI_m was determined for each group after 36 hr. It was found that increasing concentrations of EGF produce a small but significant increase in cell proliferation ($P < 0{\cdot}01$). This effect, however, was less pronounced than that seen when serum was present.

These results suggest that EGF has a trophic action on the colon and interacts with additional factors found in serum.

The existence of epidermal growth factor (EGF), a small polypeptide, in the submaxillary glands of male mice was first demonstrated by Cohen (1962) whilst studying nerve growth factor. The isolation and purification of the homogeneous peptide was later achieved (Savage & Cohen, 1972) and it was identified as a peptide consisting of 53 amino acids and three disulphide bonds (Savage, Inagami & Cohen, 1972).

Beta-urogastrone (UG), a peptide found in human urine and shown to inhibit gastric acid secretion, is thought to be the human counterpart of mouse EGF. Isolated and sequenced by Gregory (1975), UG was also found to contain 53 amino acids, 37 of which are common to both peptides. In addition, UG and EGF were shown to exhibit identical biological functions,

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including inhibition of gastric acid secretion and induction of precocious eye opening in newborn mice, and to compete for receptor sites on cultured human fibroblasts (Hollenberg & Gregory, 1976).

Human UG has been localized by immunocytochemical studies in submandibular salivary glands, duodenal Brunner's glands (Elder *et al.*, 1978; Heitz *et al.*, 1978) and more recently in several other human tissues (Kasselberg *et al.*, 1985). Body fluids, e.g. urine, plasma, milk, saliva and sweat, have also been shown to contain immunoreactive UG/EGF (Starky & Orth, 1977; Gregory, Walsh & Hopkins, 1979; Hirata & Orth, 1979). Despite the widespread distribution of both UG/EGF and its receptors in the body, the physiological role of EGF remains to be elucidated.

EGF is a highly potent mitogen and growth factor for many cell types and has been shown to stimulate cell proliferation in intact animals, organ culture and cell culture systems (Carpenter & Cohen, 1979). In particular, EGF has been shown to be trophic for the stomach and duodenum (Feldmen, Aures & Grossman, 1978), the proximal and distal small intestine and the proximal colon of 8-day-old mice (Malo & Menard, 1982), and the oxyntic mucosa of unweaned rats (Dembinski & Johnson, 1985). The premature appearance of sucrase activity in neonatal mice, together with increased activities of several other brush border enzymes along the entire small intestine, have also been reported by Malo & Menard (1982). In addition, EGF has been found to stimulate the maturation of foetal mouse duodenal absorptive cells both in organ culture (Beaulieu & Calvert, 1981) and *in utero* (Calvert, Beaulieu & Menard, 1982), whilst receptors for UG/EGF have been demonstrated on isolated rat intestinal cells (Forgue-Lafitte *et al.*, 1980; Gallo-Payet & Hugon, 1985). These findings, together with the presence of high concentrations of UG/EGF in milk (Byyny *et al.*, 1974) and its stability in an acid environment (Cohen, 1962), have led several workers to postulate a role for the polypeptide in the maturation and proliferation of stomach and small intestine in neonates.

EGF has also been reported to have trophic effects on various parts of the gastrointestinal tract of mature animals. These include, in rats, the gastric mucosa (Johnson & Guthrie, 1980; Dembinski *et al.*, 1982), duodenum (Dembinski *et al.*, 1982; Chabot, Payet & Hugon, 1983), jejunum and ileum (Chabot *et al.*, 1983; Al-Nafussi & Wright, 1982) and pancreas (Dembinski *et al.*, 1982), and, in the mouse, the tongue, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon and rectum (Scheving *et al.*, 1979; 1980). These observations suggest that EGF not only plays a part in the development of the gastrointestinal tract but may also control mucosal growth in the adult.

The development of an organ–culture system for adult rat colonic mucosa has enabled a direct assessment of the effect of EGF on cell division. The *in vitro* system has the advantage of being unaffected by some of the complex trophic interactions which occur *in vivo*. The present investigation was undertaken to examine the effect of EGF on cell proliferation with time in the presence of serum and also at various concentrations in a serum-free medium.

MATERIALS AND METHODS

Animals

Eight male Wistar Porton rats (Olac Ltd, Bicester) approximately 6 months old and weighing between 425 and 450 g were used in this study. They were maintained in standard conditions with unrestricted access to food (Breeder's Diet Number 3, Special Diet Services, Witham, Essex) and tap water.

Procedure for organ culture

Animals were killed by cervical dislocation. Immediately *post mortem* the colon was divided at the colo-caecal junction, removed, opened along its length and cleaned of faecal contents. A segment of colon approximately 100–120 mm in length was cut from a region 40 mm proximal to the anal margin. The tissue was washed gently and transported to the laboratory in ice-cold culture medium.

Using watchmaker's fine forceps the mucosa from the isolated segment was stripped from the underlying muscularis propria under stereomicroscopic examination. The mucosa was then spread luminal surface uppermost on a cork board and divided into explants of approximately 2 mm^2. The explants were placed onto cellulose acetate filters of 3·0μm pore size (Millipore (U.K.) Ltd, Harrow) within 60 mm, vented, sterile, plastic, petri dishes (Sterilin, Gibco Ltd, Paisley, Scotland). Each dish contained four explants.

The serum-containing medium employed in the first series of experiments consisted of Waymouth MB 752/1 (Gibco Ltd, Paisley, Scotland), 10% fetal calf serum (Northumbria Biologicals Ltd, Cramlington, Northumberland), 300 μg/ml ascorbic acid (Sigma London, Poole, Dorset), 3 μg/ml hydrocortisone-21-sodium succinate (Sigma London), 0·45 μg/ml ferrous sulphate (Sigma London), 100 Units/ml penicillin/streptomycin solution and 100 Units/ml mycostatin suspension (Sigma London). It should be emphasized that a single batch of serum was used throughout.

Variation in the composition of the serum from different batches and also the presence of endogenous EGF, prompted the development of a chemically-defined medium for the second series of experiments. In the past the addition of serum to the culture medium has ensured a reliable long-term survival of intestinal mucosa. In our serum-free medium therefore, the following supplements were added at levels designed to enhance tissue preservation; sodium butyrate 1 mM (BDH Chemicals Ltd, Poole, Dorset), 50 ng/ml glucagon, 5 μg/ml transferrin, 5 μg/ml insulin (all Sigma London except where stated).

The pH of the medium was adjusted to 7·4 if required and was filter sterilized through a 0·22 μm filter unit (Millipore (U.K.) Ltd., Harrow). Each petri dish received 2·5 ml of freshly prepared medium. The cultures were maintained at 37°C within a humidified controlled atmosphere chamber (Bellco Glass Ltd, Vineland, New Jersey) and rocked at eight cycles per minute on a rocking platform (Bellco Glass Ltd). The medium was changed every 48 hr and the gas phase (95% O_2/5%CO_2) renewed daily.

Investigation of the effect of EGF with time

Explants of colonic mucosa from four animals were maintained in the medium containing serum for five days to allow cell proliferation within the tissue to stabilize. On the fifth day of culture half the explants received fresh medium containing EGF (Sigma London) at a concentration of 40 ng/ml and the remainder (controls) fresh medium only. At 6, 12, 24 and 48 hr both experimental and control explants received vincristine (Oncovin, Eli Lilly, Basingstoke) at a dose of 4 μg/ml for 3 hr prior to fixation in neutral buffered formalin. After fixation the tissue was dehydrated through changes of cellosolve to chloroform and processed in paraffin wax. Transverse serial sections, 4 μm thick, were stained with haematoxylin and eosin.

Investigation of the effect of EGF at various concentrations

In a second series of experiments colonic explants from four animals were maintained for five days in the serum-free medium. The explants were then divided into 12 equal groups and received either fresh medium only (controls), medium containing 10% fetal calf serum, or medium containing one of a range of concentrations of EGF (0·1, 1, 2·5, 5, 10, 20, 40, 80, 160 or

200 ng/ml). After 36 hr each group received vincristine (4 μg/ml) for 3 hr prior to fixation. The tissue was processed as described and the transverse sections of 4 μm thickness were cut and stained with haematoxylin and eosin.

Counting procedures

The metaphase-arresting drug vincristine has been employed to identify any changes in proliferation which may occur on the addition of EGF. The proportion of cells which had divided within each explant during the 3-hr period of its addition was determined and expressed in the form of a vincristine-augmented mitotic index (AI_m). The native mitotic index (I_m) for colonic mucosa *in vitro* was too low to be used to detect differences in cell division between treated and control tissues. An AI_m, however, was able to accentuate changes in proliferation and provide both a simple and reliable index for cultured tissue.

A previous study (Finney *et al.*, 1987) of proliferation at the edge and centre of cultured explants indicated that kinetic parameters in these two regions differed markedly. The cell birth-rate at the edge (7·5 $\pm$ 0·9 cells/1000 cells/hr), although very similar to the *in vivo* value (7·8 $\pm$ 0·8 cells/1000 cells/hr), was approximately twice that found at the centre (3·2 $\pm$0·9 cells/1000 cells/hr). This suggests that crypts at the edge of the explant are already stimulated and are of only a limited usefulness when studying responses to trophic factors, whereas the crypts at the centre of the explant provide a more basal level of proliferation from which to monitor trophic responses. Therefore, in the light of these findings, the edges, arbitrarily defined as the most peripheral three crypts, were excluded from the cell counting.

Vertical sections were cut for each explant; however, the natural curvature and small size of the explant made it very difficult to obtain perfect axially sectioned crypts. Therefore, to obtain an AI_m, the total number of crypt epithelial cells in the middle region of the explant was counted, and the number of arrested metaphases was noted. Serial sections were employed in whatever number necessary to obtain a minimum of 1000 cells. In order to obviate disturbances in proliferation caused by tissue degeneration, only explants showing good tissue preservation and near normal morphology were included in the counting.

RESULTS

Morphology of cultured explants

Sixty-six per cent of the explants maintained in serum-containing medium and 62% of those in a serum-free medium showed morphological preservation judged to be good enough to be counted. There was no appreciable morphological difference between explants maintained in either medium (Fig. 1a, 1b).

For the first series of experiments the addition of EGF at a concentration of 40 ng/ml for 6, 12, 24 and 48 hr did not change explant viability. However, at 24 hr, and more noticeably at 48 hr, there appeared in treated explants to be a reduction in the number of goblet cells present in each crypt together with an increase in cell shedding as indicated by the presence of pyknotic nuclei around the luminal surface (Fig. 2).

The addition of EGF for 36 hr to explants maintained in a serum-free medium appeared not to cause any consistent alteration in morphology or preservation at any concentration. At EGF dose levels of 160 and 200 ng/ml, in one of the four experiments, loss of goblet cells and increased cell shedding were again apparent (Fig. 3). These changes, however, were seen consistently when serum was added to explants for a 36-hr period. The short-term addition of serum, whilst leading to increased cell division (see below), appeared also to have a deleterious effect on preservation, often leading to focal areas of cell loss and necrosis (Fig. 4).

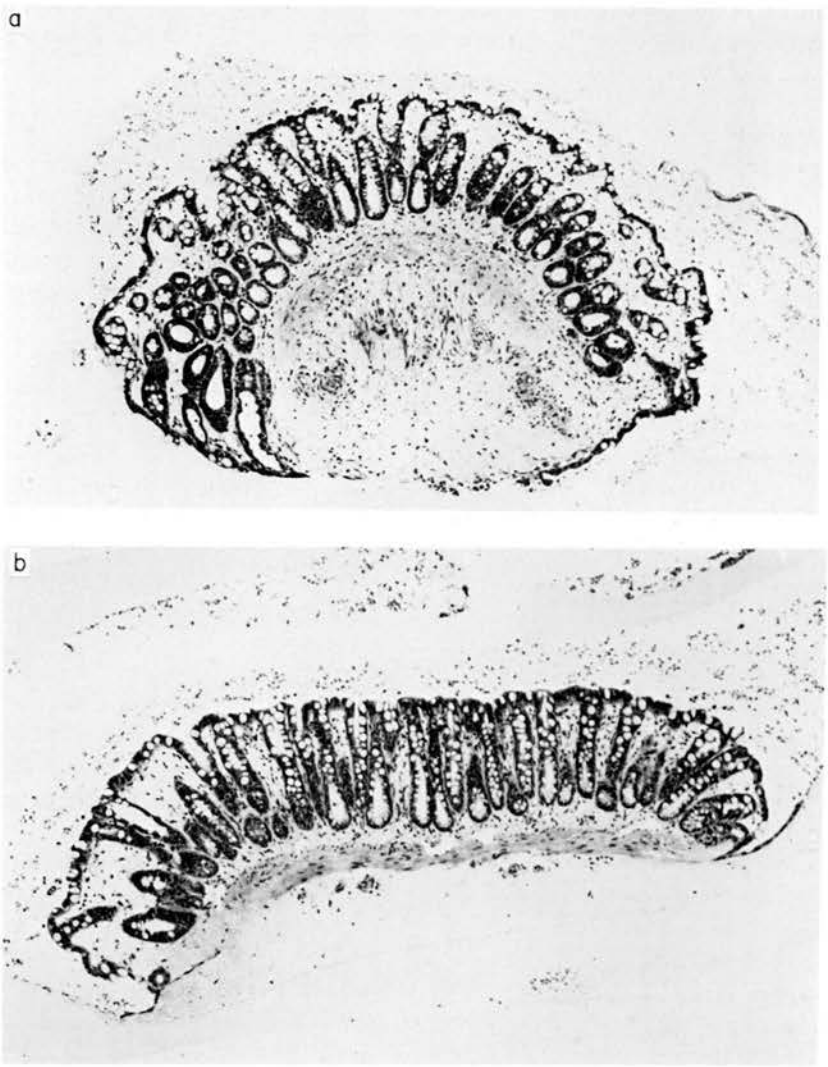

Fig. 1. Explant of colonic mucosa after 7 days culture in (a) medium containing serum, (b) serum-free medium. Haematoxylin & Eosin (×110).

Effect of EGF with time in the presence of serum

The original data for each of the four animals, together with lines to represent the combined values for treated and control groups at each timepoint, are presented in Fig. 5. The mean AI_m, together with its standard error for the pooled data, is given in Table 1.

Figure 6 shows a transformation of the data to achieve linearity. The ratio of the AI_m (EGF) to the AI_m (CONTROL) for each animal has been plotted against time and these data have been represented by four parallel lines and the equation:

$$\frac{AI_m(\text{EGF})}{AI_m(\text{CONTROL})} = k + 0{\cdot}0426 \times t,$$

where k is a constant and t is the time in hours.

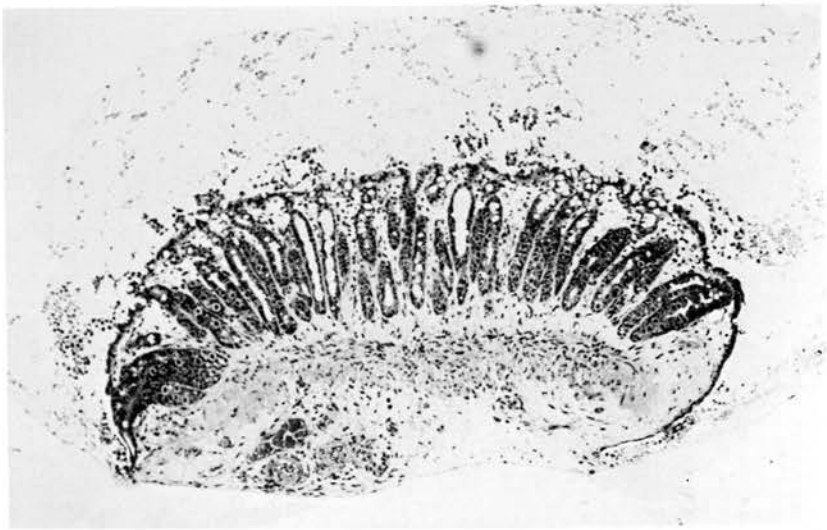

Fig. 2. Appearance of a 7-day-old explant after the addition of EGF (40 ng/ml) for 48 hr to a medium containing serum. Haematoxylin & Eosin (× 110).

Fig. 3. Appearance of a 7-day-old explant after the addition of EGF (200 ng/ml) for 36 hr to a serum-free medium. Haematoxylin & Eosin (× 110).

Each line can be seen to have a different value for the intercept. This is best explained by both animal and explant variation. Despite this fact, however, each replicate appears to be affected by the addition of EGF to the same degree.

An analysis of variance was used to test whether the slope was significantly different from zero. The standard error of the slope was found to be 0·0081 to give $t = 5{\cdot}23$. This result indicates that there is an effect of EGF and that it increases with time ($P < 0{\cdot}001$).

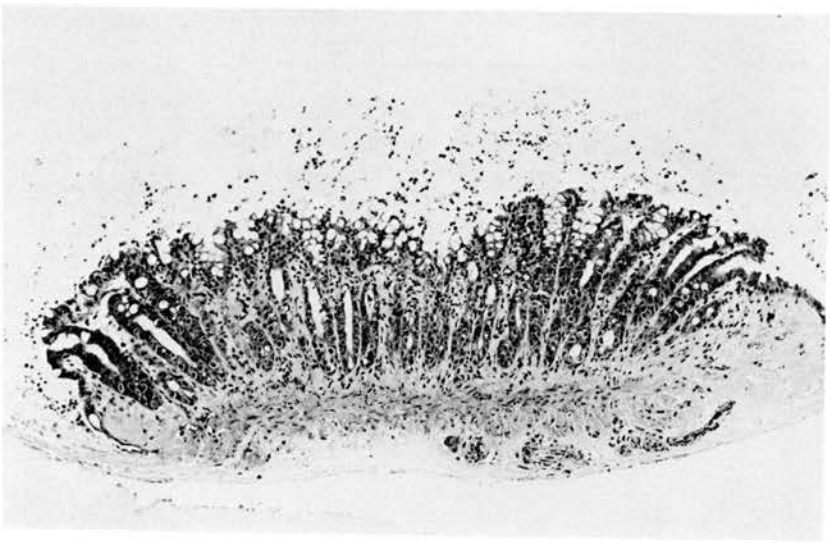

Fig. 4. Explant of colonic mucosa, initially maintained in a serum-free medium, after the addition of serum for a 36-hr period to show an increased number of arrested metaphases and areas of necrosis. Haematoxylin & Eosin (×110).

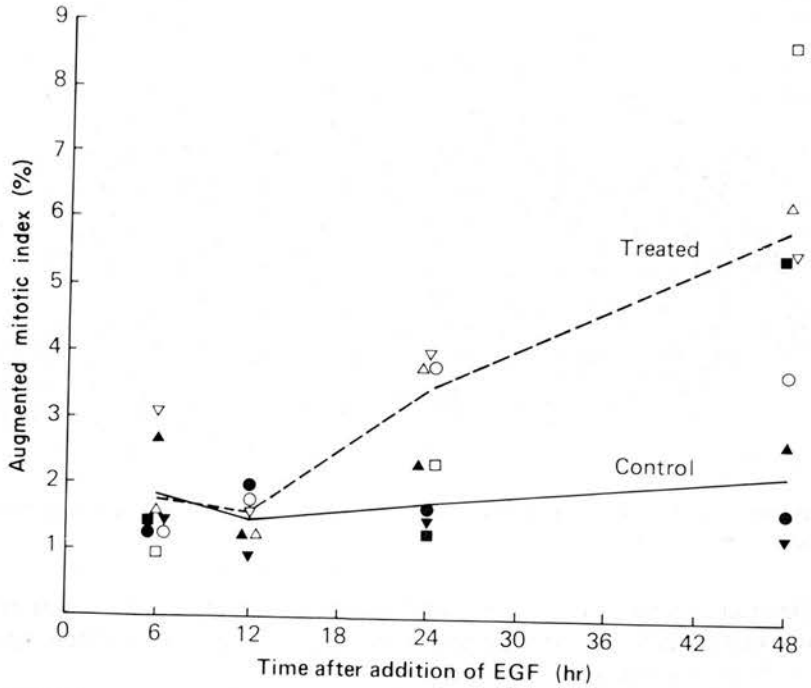

Fig. 5. Effect of EGF (40 ng/ml) in the presence of serum. The original data points for each animal (open symbols—EGF treated, closed symbols—controls) are shown. Replicates (circles) = 1, (squares) = 2; (triangles) = 3; (inverted triangles) = 4. $P < 0{\cdot}001$.

Table 1. Effect of EGF with time in a medium containing serum

	AI_m(%)	
Time (hr)	Control	EGF
6	1·92 (0·18)	1·86 (0·15)
12	1·42 (0·16)	1·52 (0·12)
24	1·77 (0·14)	3·56 (0·23)
48	2·37 (0·34)	6·04 (0·40)

Mean AI_m(%) together with standard errors are given for both control and treated groups.

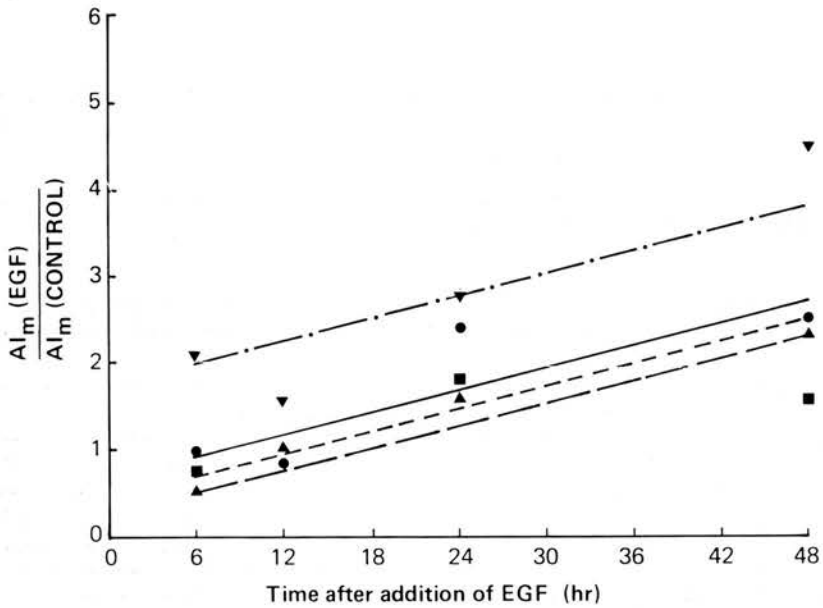

Fig. 6. Effect of EGF (40 ng/ml) in the presence of serum. Linear transformation of the data has been achieved by plotting the ratio AI_m EGF : control along the y axis. Replicates: (●——) = 1; (■— —) = 2; (▲– – –) = 3; (▼—·) = 4.

Effect of EGF at various concentrations in a serum-free medium

Figure 7 shows the spread of the raw data for the four animals employed in the serum-free experiments. The AI_m after 36 hr has been plotted against the logarithm (base 10) of the concentration of EGF. Both control and serum groups have been placed on this graph to enable a comparison with the EGF-treated groups. It appears that as the dose of EGF increases there is a slight upward trend in the AI_m. Clearly, the addition of serum for 36 hr produces a marked increase in cell proliferation ($P < 0{\cdot}001$).

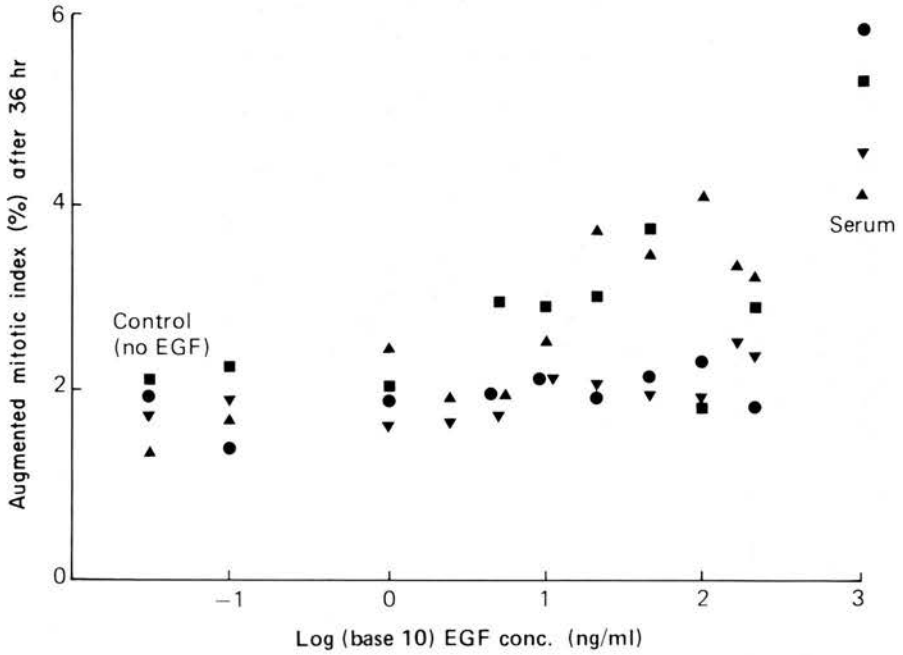

Fig. 7. Effect of EGF in a serum free medium, to show the spread of original data for the individual animals. Replicates: (●) = 1, (■) = 2; (▲) = 3; (▼) = 4.

Table 2. Effect of EGF at various concentrations in a serum-free medium

		AI_m(%)									
		EGF (ng/ml)									
Control	Serum	0·1	1	2·5	5	10	20	40	80	160	200
1·79 (0·01)	5·25 (0·30)	1·86 (0·14)	2·01 (0·14)	1·81 (0·11)	2·07 (0·17)	2·38 (0·12)	2·63 (0·17)	2·88 (0·22)	2·40 (0·24)	2·87 (0·27)	2·49 (0·21)

The mean AI_m(%) together with standard errors are given for each group.

The relationship between the AI_m and dose of EGF has been analysed using the computer program GLIM (Baker & Nelder, 1978). In order to linearize the data, the AI_m for each of the four experiments was transformed to the logit

$$\text{logit } AI_m = \log_e\left(\frac{AI_m}{1 - AI_m}\right)$$

and plotted against the logarithm (base 10) of the concentration of EGF. Curves fitted by the logistic transformation for each experiment are shown in Fig. 8a. Computer analysis indicates that these lines can be considered as parallel without any significant loss of fit, the value of the slope is 0·1254 (Fig. 8b). An analysis of variance gives a standard error of 0·0127 with $t = 2{\cdot}70$ to indicate that as the concentration of EGF increases there is a small but significant rise in cell proliferation ($P < 0{\cdot}01$).

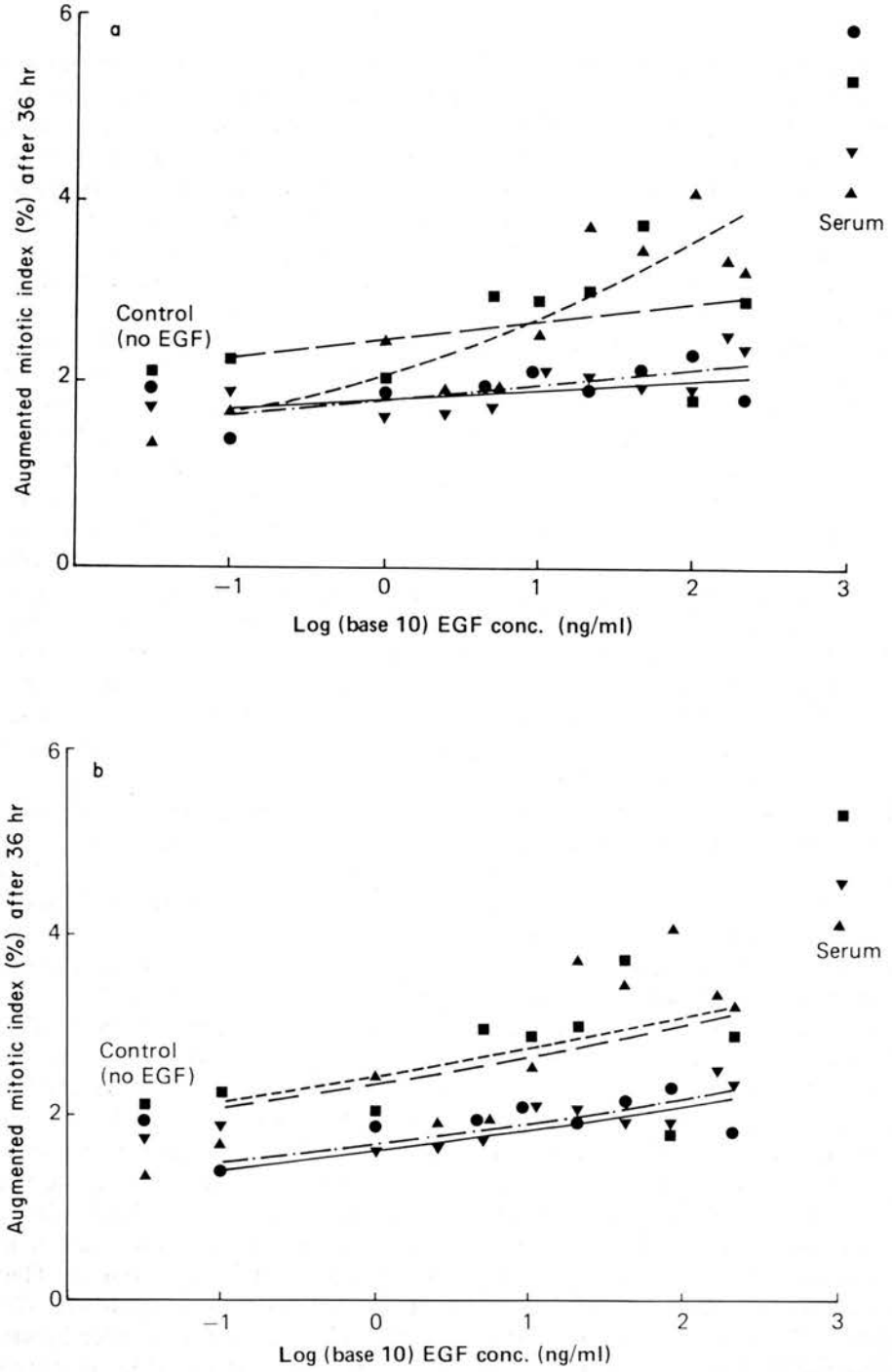

Fig. 8. Effect of EGF in a serum-free medium: (a) curves have been fitted to individual experiments using a logit transformation of the AI_m; (b) a single curve has been fitted for all four experiments ($P < 0{\cdot}01$). Replicates: (●—) = 1; (■——) = 2; (▲---) = 3; (▼-·-) = 4.

DISCUSSION

In the first series of experiments, the addition of exogenous EGF (40 ng/ml) in the presence of serum was found to be effective in increasing cell proliferation in explants of colonic mucosa. This effect was found to increase with time and was statistically significant (Fig. 5). The AI_m for the EGF-treated group was approximately twice that of the control group at 24 hr and almost three times as great at 48 hr. The lag phase shown by the colonic mucosa, between the addition of EGF and the appearance of an increase in cell division, would seem to be in agreement with that noted for several cell types in culture. However, it is highly likely that the movement of stimulated cells through G_1, S and G_2 constitutes some of this apparent lag phase and that EGF probably exerts its effective proliferation signal at a much earlier time.

Complex changes are known to occur after the stimulation of quiescent cells by polypeptide growth factors or serum and these include both 'early' and 'late events'. Early events involve changes in nutrient and ion transport and increased membrane activity in the form of ruffling and macropinocytosis, and generally occur within 1–2 hr after the addition of the stimulus. Within 12 hr an increase in RNA and protein synthesis is seen, this leads to the initiation of DNA synthesis and increased cell proliferation after 10–15 hr (Carpenter & Cohen, 1979).

Despite the fact that many polypeptide growth factors, including platelet-derived growth factor (PDGF), fibroblast-derived growth factor (FDGF), fibroblast growth factor (FGF) and EGF, have been shown to regulate the proliferation of normal cells (Rozengurt & Collins, 1983), the exact mechanism leading to the mitogenic response is poorly understood. The importance of the internalization of the growth factor–receptor complex in the elicitation of mitogenesis has become a matter for debate. Shechter, Hernaez & Cuatrecasas (1978) have shown that the biological activity of EGF requires the persistent occupation of high-affinity cell surface receptors. The addition of anti-EGF IgG after 8 hr exposure to the polypeptide can completely reverse its effect on cell proliferation.

The concentrations of EGF employed in the second series of experiments performed in a serum-free medium were intended to represent those concentrations reported to occur in body fluids: plasma (1–2 ng/ml), saliva (5–17 ng/ml), milk (80 ng/ml) and urine (29–272 ng/ml) (Starkey & Orth, 1977). However, the actual concentration of EGF at the cell surface in the gastrointestinal tract is difficult to estimate.

Although a small but significant rise in the AI_m was produced as the concentration of EGF was increased, the most marked increase in cell proliferation occurred on the addition of serum for 36 hr. Serum has been regarded as an essential requirement for the growth and proliferation of cells in culture. It provides hormones, nutrients, binding proteins which may detoxify the medium, protease inhibitors, and acts as a buffer for the pH (Barnes & Sato, 1980). Addition of serum to quiescent cells results in a stimulation of DNA synthesis and cell division.

In contrast, it is interesting to note that the levels of cell proliferation seen in the control explants maintained in a serum-free medium are very similar to those seen for the controls maintained in the presence of serum throughout the culture period. Thus, whilst the short-term addition of serum results in a large increase in cell proliferation, the presence of serum for longer periods of time may cause the cells to become 'desensitized' to its mitogenic stimulus. This may occur by a 'down-regulation' of specific receptors or by a block at an intracellular level. Clearly, endogenous EGF alone is not responsible for the marked stimulation of cell division by serum as the addition of EGF at serum levels (1–2 ng/ml) in a serum-free medium produced only a small increase in the AI_m.

The addition of 40 ng/ml EGF to explants in a serum-free medium produced a mean AI_m that was approximately only half the value seen when serum was present. This indicates that EGF

interacts with additional growth factors found in the serum to lead to enhanced levels of proliferation. Bertsch & Marks (1974) reported that EGF strongly stimulated DNA synthesis in explants of chick embryo epidermis in a serum-free medium and that responsiveness of the tissue to the hormone was increased by fetal calf serum. EGF has also been shown to stimulate DNA synthesis in quiescent cultures of 3T3 cells in serum-free medium. However, in other cell types the activity of EGF is dependent upon the presence of a small concentration of serum (Rozengurt, 1980). Mierzejewski & Rozengurt (1978) have partially purified a polypeptide found in serum which acts synergistically with EGF and insulin and is as effective as whole serum in the stimulation of DNA synthesis in 3T3 cell cultures.

An increase in cell shedding was observed at 48 hr after the addition of EGF in the presence of serum and also on one occasion at EGF dose levels of 160 and 200 ng/ml in a serum-free medium. Although no attempt has been made to identify which of the kinetic parameters is altered by the addition of EGF, it is likely that this increase, taken together with an increase in the AI_m and a loss of differentiated (goblet) cells in both instances, reflects an increased rate of cell turnover with a reduced cell transit time.

EGF has been reported to have a trophic effect on colonic mucosa *in vivo*. Scheving *et al.* (1980) found that EGF increased DNA synthesis in the mouse colon and maximal stimulation occurred at 8 and 12 hr post injection. Al-Nafussi & Wright (1982) observed that although EGF stimulated colonic crypt cell proliferation in mice 8 hr after injection is was not effective for the colon of rats. Other workers, however, have found no effect of EGF on the colon of mice (Feldman *et al.*, 1978) or rats (Johnson & Guthrie, 1980). DNA synthesis in the gastrointestinal tract exhibits circadian variation and the stimulatory effect of EGF has been shown to depend upon the particular time at which it is administered *in vivo* (Scheving *et al.*, 1979; 1980). Failure to consider this fact could lead to a conflict of results in intact animals.

Chabot *et al.* (1983), using an organ culture system for duodenal and jejunal explants, did not find an increase in DNA synthesis after treatment with a range of concentrations of EGF in a medium either with or without serum. These workers concluded that the stimulation of DNA synthesis in the small intestine *in vivo* was not a direct effect of EGF upon the gut mucosa.

We have shown that EGF does have a direct effect on the cell proliferation of colonic mucosa in organ culture. It is probable that the effect of EGF, and indeed the extent of the effect, will depend upon the general state of proliferation within the tissue, the availability of specific nutrients and the integrity of the cell's receptors. The effect of EGF was, for example, found to be more pronounced in the presence of serum. It could be envisaged that *in vivo* an intrinsic basal steady-state of proliferation occurs in the gut which is periodically modulated by a complex interaction of extrinsic factors that include polypeptides such as EGF.

ACKNOWLEDGMENTS

This work was supported by a grant from the North of England Cancer Research Campaign (NECC 418021). We would like to thank Kathryn Elliott for her technical assistance, Mr S. Brabazon, who prepared the photographs, and Miss E. Wark for typing the manuscript.

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THE EFFECT OF TESTOSTERONE ON CELL PROLIFERATION AND DIFFERENTIATION IN THE SMALL BOWEL

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J. Endocr. (1972), **52**, 161–175
Printed in Great Britain

THE EFFECT OF TESTOSTERONE ON CELL PROLIFERATION AND DIFFERENTIATION IN THE SMALL BOWEL

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(*Received* 21 *June* 1971)

SUMMARY

The action of testosterone on the cell kinetics of the small bowel was studied in the castrated male mouse. The parameters of the cell cycle were measured using the labelled mitoses method. No difference was found between cell cycle parameters in testosterone-treated castrated animals compared with castrated controls.

Crypt cell kinetics were studied by measuring the distribution of labelled and mitotic nuclei using a computer programme. The labelling and mitotic indices were significantly raised in the testosterone-treated animals. There was also a significant upward displacement of the cut-off position in the testosterone-treated group, indicating an increase in the size of the proliferative compartment, and thus an increase in growth fraction. This change in growth fraction was confirmed by calculation from the labelled mitoses results, and is considered to be the mechanism by which testosterone stimulates cell proliferation in the small bowel of the castrated mouse. The action of testosterone on the growth fraction may constitute an important component of the general mitogenic effect of the hormone on both target and non-target tissues.

INTRODUCTION

The mitogenic effect of testosterone on the accessory sex organs of the castrated animal is now well established (Allen, 1958). Further studies have demonstrated that testosterone has profound effects on the duration of cell cycle parameters in these tissues both *in vivo* and *in vitro* (Tuohimaa & Niemi, 1968; Morley, 1970). Recent work has indicated that androgens also affect cell kinetics in several non-target tissues. Testosterone has been shown to stimulate DNA synthesis in bone marrow (Diamond, Jacobson & Sidman, 1967) and has been used with some success in the treatment of aplastic anaemia. However, it has been suggested that the erythropoietic action of testosterone is mediated by erythropoietin (Rencricca, Solomon, Fimian, Howard, Rizzoli & Stohlman, 1969).

The small intestine is a non-target organ where testosterone has been shown to

stimulate cell proliferation (Tuohimaa & Niemi, 1968). The study of cell proliferation in the small bowel has particular advantages. The duration of cell cycle components can be obtained with ease, and direct measurement of growth fraction and of transit times through the various kinetic compartments (Quastler & Sherman, 1959) are possible. Furthermore, information concerning the rate of cell differentiation can be obtained (Cairnie, Lamerton & Steel, 1965*a*). In this paper we attempt to explain the mechanism of action of testosterone on an important cell renewal system.

MATERIALS AND METHODS

Male Balb/c mice were castrated at the age of 3 months using a transperitoneal approach under ether anaesthesia. Fourteen days later 250 μg testosterone propionate (Koch–Light, Colnbrook, Bucks., England) dissolved in sesame oil were administered subcutaneously daily for 3 consecutive days. Control animals were given sesame oil subcutaneously over a similar period.

Tritiated thymidine ([^{3}H]Tdr) (Radiochemical Centre, Amersham, England) of specific activity 5 Ci/mM was administered in a dosage of 1 μCi/g body weight by intraperitoneal injection. The [^{3}H]Tdr was given at the same time as the third injection of testosterone or sesame oil. The concentration of [^{3}H]Tdr used was 100 μCi/ml. All injections were carried out at 09.00 h.

Animals were killed by cervical dislocation. Samples of upper jejunum were fixed in Carnoy's fluid for 5 h. Transverse sections were cut at a thickness of 3 μm, exposed for 14 days using Kodak A.R.10 and developed with Kodak D19b. The preparations were then fixed, washed and stained with Harris's haematoxylin.

Experiment 1. Testosterone-treated and control animals were killed serially from 0·5 to 48 h after a single injection of [^{3}H]Tdr. Crypts were selected in which the base, lumen and top of the crypt were present in the plane of section. The incidence of labelled metaphases and anaphases was determined for each time-period. At least 100 metaphases and anaphases were counted for each animal. Prophases and telophases were excluded because of difficulties in recognition, and also to decrease the size of the labelled cohort. The incidence of labelled mitoses was plotted against time. The median durations of cell cycle parameters were measured from the labelled mitoses curve at the %50 level (Quastler & Sherman, 1959).

Abbreviations used are as follows: t_{g2}, the duration of the premitotic interval. Since prophases were not counted, this is strictly t_{g2} + the duration of prophase. t_m, the duration of mitosis; t_{g1}, the duration of the presynthetic interval; t_s, the duration of interphase DNA synthesis; T_c, the duration of the cell cycle, or cell cycle time.

Experiment 2. Five castrated, testosterone-treated and five castrated control mice were killed 1 h after administration of [^{3}H]Tdr. In each animal 30 crypts were selected. Care was taken to ensure that each crypt was analysed only once. The counting techniques of Cairnie *et al.* (1965*a*) were used throughout. In each crypt the left-hand column of cells was numbered, counting from the bottom upwards to the crypt–villus junction. The positions of labelled and mitotic nuclei were recorded. In this experiment all stages of mitosis were included. A mitosis situated midway between the right and left hand columns was counted as a half (Cairnie

et al. 1965*a*). A cell was considered to be labelled if six or more grains were present over the nucleus. Background was usually negligible.

The variation in the height of crypt columns is shown for the experimental group in Fig. 1. It is evident that there was considerable spread in crypt height. Cairnie & Bentley (1967) found considerable differences in the distribution of labelled cells in short and long crypts of normal animals if the positions of cells were taken relative to the top or bottom of the crypt. For this reason we analysed the results by a modified version of the method used by Cairnie & Bentley (1967). This method uses a large 'standard' crypt.

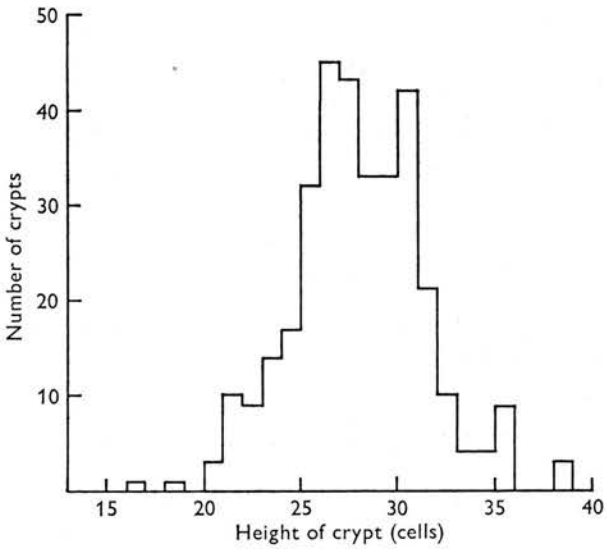

Fig. 1. Distribution of crypt height in the jejunum of castrated testosterone-treated mice. There were 150 crypts in the sample.

The results were analysed by an ALGOL programme run on an IBM 360/67 computer which produced graphical output. Let a be the number of cells in the standard crypt. This was taken to be 1000 for the results given. The standard crypt was represented by an array A with a elements 1, 2, . . ., a. Originally each element of A is set to zero.

Consider an actual crypt of size b with a cell in position r to which a score k was assigned. For example $k = 1$ for a labelled cell, and 0 for an unlabelled cell, with extensions for cells in mitosis where k may be 0, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, etc. This latter extension is required since mitoses may be found midway between the two crypt columns (see above) and more than one mitosis can be present opposite the same cell position. Elements A_i are then increased by an amount $k \times b$ for each of the a/b cells, taking values of i from

$$i = \frac{a}{b(r-1)} \quad +1 \quad \text{to} \quad i = \frac{a \times r}{b}.$$

This partitions the a cells of the standard crypt into equal lengths of a/b cells.

This method ensures that the total added is the same, i.e. a, for a labelled cell in a

crypt of any size. In Cairnie & Bentley's method there is a bias towards the distribution in small crypts which is avoided by the technique described.

Results are printed corresponding to a crypt of any size p. The value chosen for p is conveniently the mean crypt size if crypts of similar sizes are being compared; if one is dealing with crypts containing widely differing numbers of cells it is easy to look at results at the mean crypt size of either set. The labelling (or mitotic) index for cell j of a crypt of size p, if there are d crypts altogether, (LI_j) is calculated by:

$$LI_j = \frac{100}{da} \Sigma A_i, \tag{1}$$

where i takes values from

$$\frac{a}{p(j-1)}+1 \quad \text{to} \quad \frac{a \times j}{p}.$$

Approximate 95% confidence limits for the labelling index of each cell position are produced assuming that LI_j is binomially distributed.

RESULTS

Crypt height

The mean crypt height in the castrated control animals was $27{\cdot}0 \pm 4{\cdot}0$ (S.D.) cells, and $27{\cdot}4 \pm 3{\cdot}5$ in the castrated, testosterone-treated group. The difference was not significant.

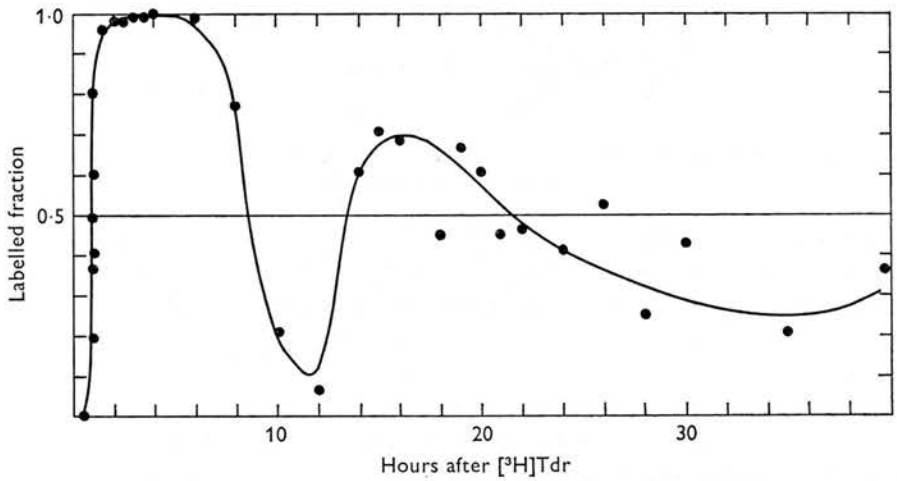

Fig. 2. Fraction of labelled mitoses in the jejunal crypts as a function of time after pulse labelling of animals with tritiated thymidine ([³H]Tdr) in castrated (control) mice.

Cell cycle parameters

The percentage labelled mitoses curve for the castrated controls is shown in Fig. 2, and for the castrated testosterone-treated group in Fig. 3. The duration of cell cycle parameters is given in Table 1; t_{g2} was found to be 1 h in each group, t_s was decreased from 7·8 h in the control group to 6·6 h in the testosterone-treated group, t_{g1} was fractionally less (3·6 h) in the controls compared with the testosterone-treated

animals (3·8 h), T_c was 13·4 h in the control, and 12·4 h in the testosterone-treated groups respectively. It is evident that this difference is due predominantly to a decrease in t_s in the experimental group. Baserga & Malamud (1969) state that significance should not be implied unless differences of at least 20 % are obtained in labelled mitoses data. We conclude therefore that no real difference exists between the two groups.

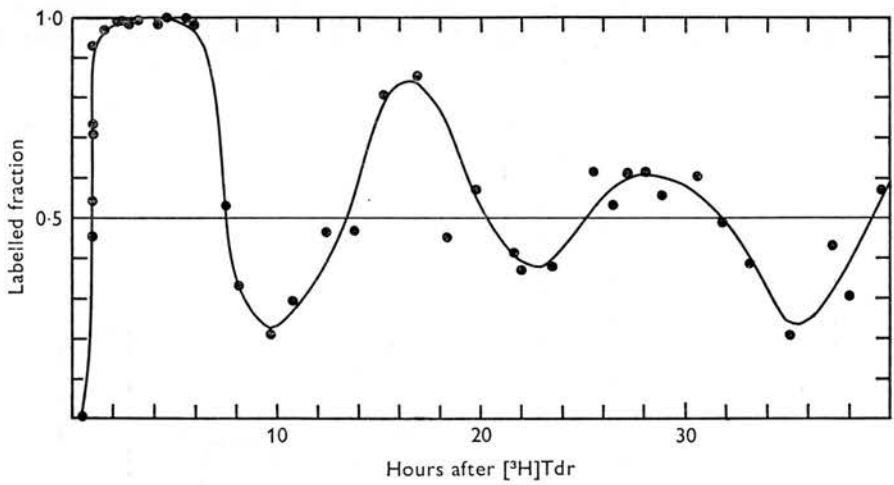

Fig. 3. Fraction of labelled mitoses in the jejunal crypts as a function of time after pulse labelling of animals with tritiated thymidine ([³H]Tdr) in castrated testosterone-treated mice.

Table 1. *Duration in hours of cell cycle parameters in the small intestinal crypts of testosterone-treated and castrated male Balb/c mice*

(For definitions of t_s, etc., see text, p. 162.)

	t_s	t_{g2}	t_m*	t_{g1}	T_c	LI (%, mean ± S.D.)	MI (%)†	Crypt height (cells ± S.D.)
Castrated animals (controls)	7·8	1·0	1·0	3·6	13·4	23·4 ± 1·3	3·0	27·0 ± 4·0
Testosterone-treated, castrated animals	6·6	1·0	1·0	3·8	12·4	37·4 ± 2·0	5·3	27·4 ± 3·5
P value	—	—	—	—	—	< 0·001‡	< 0·001§	NS‡

* t_m was assumed to be 1·0 h in each case.
† S.D. not given since distribution of mitotic cells in the crypt was not normal.
‡ Submitted to Student's *t*-test. NS = not significant.
§ Submitted to a median test (see text, p. 167).

The measurement of t_m from labelled mitoses curves is difficult, especially in populations where t_{g2} is short. Assuming steady state conditions a value for t_m can be obtained by calculation from the expression

$$\frac{t_m}{t_s} = \frac{n_m}{n_s}, \tag{2}$$

where n_m and n_s are the number of dividing and labelled cells respectively. From this, t_m in the controls was 1·0 h and in the testosterone-treated group 0·9 h. How-

ever, there are a number of serious objections to this method of determining the duration of mitosis (see Discussion), and where later calculations involve t_m this is taken to be 1 h in each group (Cairnie *et al.* 1965*a*; Stevens Hooper, 1961).

Labelling index and mitotic index

The labelling index (LI) in the castrated controls was $23{\cdot}4 \pm 1{\cdot}3$ (S.D.) % and in the castrated testosterone-treated animals $37{\cdot}4 \pm 2{\cdot}0$ % (Fig. 4). The difference was highly significant ($P < 0{\cdot}001$). The mitotic index (MI) was 3·0 % in the controls and

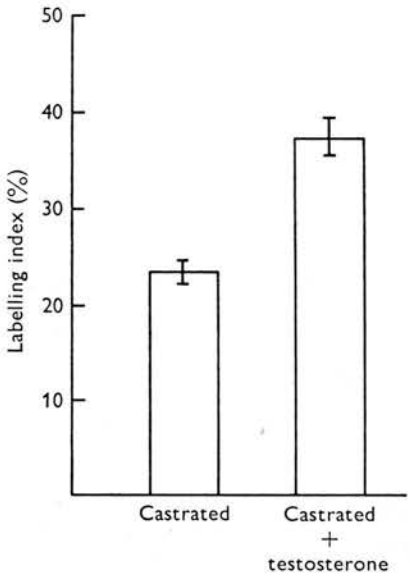

Fig. 4

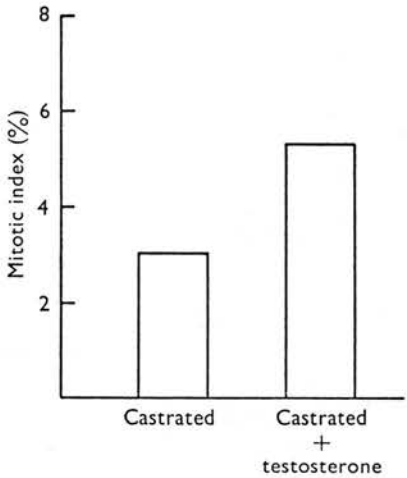

Fig. 5

Fig. 4. Labelling index (% of cells labelled) in the jejunal crypts of castrated mice (controls) compared with castrated testosterone-treated animals ($P < 0{\cdot}001$, Student's *t*-test). The vertical bars indicate ±S.D.

Fig. 5. Mitotic index (% of cells in mitosis) in the jejunal crypts of castrated mice (controls) compared with castrated testosterone-treated animals ($P < 0{\cdot}001$, median test).

5·3 % in the testosterone-treated groups respectively (Fig. 5). Figures 6 and 7 show the number of mitoses per crypt column for each group. In each case there is close agreement to a Poisson distribution. The goodness of fit was tested by the variance test for the Poisson distribution. For the controls the 95 % confidence limits for the mean were 0·69 and 0·99 and the probability that the distribution was not a Poisson was $P < 0{\cdot}1$. In the case of the testosterone-treated group the 95 % confidence limits for the mean were 1·18 and 1·49, and the probability that the distribution was not a Poisson was $P < 0{\cdot}05$. The difference between the two means was highly significant ($P < 0{\cdot}001$).

If crypt cells were asynchronous with respect to phase in the cell cycle, mitoses would show a Poisson distribution. If cells showed synchrony to any great extent many crypts would have no mitoses, and a few crypts with many mitoses would be

evident (Cairnie *et al.* 1965*a*). Synchrony would have important repercussions on the measurement of cell cycle parameters from the labelled mitoses curve. Since the distribution of mitoses was not normal, the median test was used to see whether the two samples could have been taken from the same population. The median observation was 3·7; $\chi^2 = 18{\cdot}32$; D.F. = 1; $P < 0{\cdot}001$. This confirms the difference in MI between the testosterone-treated and control animals.

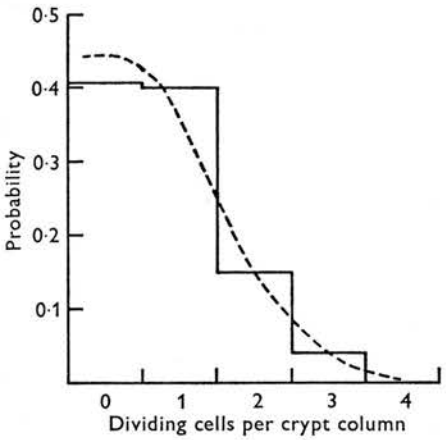

Fig. 6

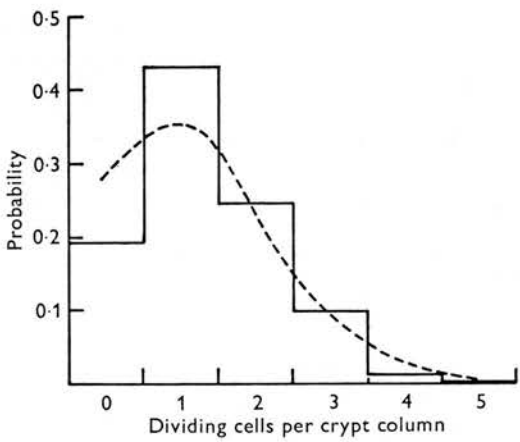

Fig. 7

Fig. 6. Number of mitoses per crypt column in the jejunum of castrated mice (controls). The curve indicates a Poisson distribution with a mean of 0·80.

Fig. 7. Number of mitoses per crypt column in the jejunum of castrated testosterone-treated mice. The curve indicates a Poisson distribution with a mean of 1·35.

The growth fraction

The growth fraction (GF) is the fraction of a particular population that consists of cells progressing round the cell cycle. This can be determined experimentally from the ratio

$$\mathrm{GF} = \frac{\mathrm{LI_{expt}}}{\mathrm{LI_{theor}}}, \tag{3}$$

where $\mathrm{LI_{expt}}$ is the observed LI, and $\mathrm{LI_{theor}}$ is the theoretical LI which would be expected if all cells were in the cell cycle. The theoretical LI can be obtained, assuming steady state conditions, from the expression

$$\mathrm{LI} = \frac{t_s}{T_c}, \tag{4}$$

since T_c and t_s are known from the labelled mitoses data (Table 1). Thus calculated, the GF is 0·40 in the control and 0·70 in the testosterone-treated animals respectively. This suggests that the cause of the increased LI, in the absence of a reduction in T_c, is a raised GF in the testosterone-treated group. It should be emphasized, however, that this calculation is based on the assumption of steady state conditions, which may not be applicable, particularly in the testosterone-treated group.

Distribution analysis

Figs 8 and 9 show the crypt distribution curves for LI and MI respectively in the two groups. In the control group the LI remained between 30 and 40 % in the first four cell positions, rising to a maximum value of near 50 % at positions 6–10. The

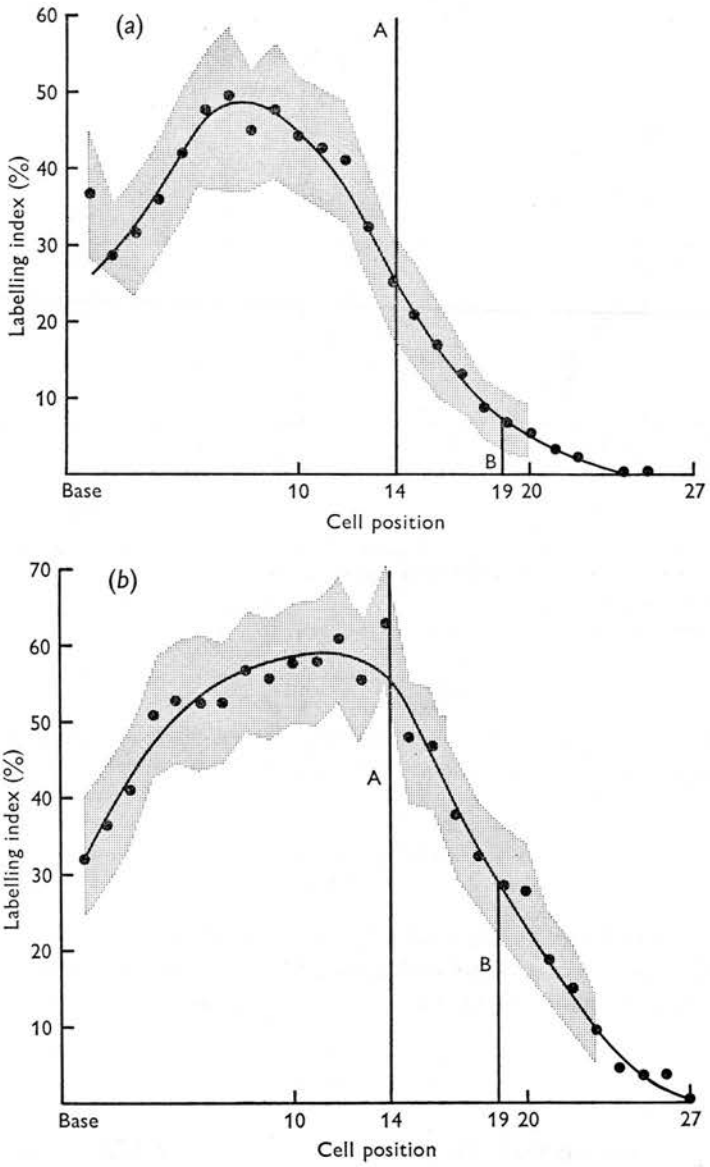

Fig. 8*a* and *b*. Distribution of labelling indices in jejunal crypts of mice calculated on a cell positional basis. The upper curve (*a*) is for the castrated (control) group, and the lower (*b*) for the castrated testosterone-treated animals. The shaded areas indicate 95 % confidence limits of the observations. The perpendicular line A is drawn at the cell position of the 50 % peak value for the controls, and the line B in the same position for the testosterone-treated animals. These values were 14·2 and 18·7 respectively.

LI then fell to below 20 % at position 16. In the testosterone-treated group, although a similar curve was obtained, the plateau peak approached 60 %. The trailing edge was less precipitous so that the cells towards the top of the crypt had a higher LI compared with the controls. The fraction of the crypt occupied exclusively by the proliferative compartment was the region over which labelled cells were observed. This region was taken to end approximately at the cell position in the standardized crypt where the LI fell to 50 % of the maximum or peak value (Cleaver, 1967). The perpendicular lines A and B in Fig. 8 are drawn at the position of the 50 % peak

Table 2. *The growth fraction (GF) in the small intestinal crypts of testosterone-treated and castrated Balb/c mice*

	Calculated GF assuming steady state	GF measured from labelling index distribution curve
Castrated animals (controls)	0·40	0·52
Testosterone-treated, castrated animals	0·70	0·69

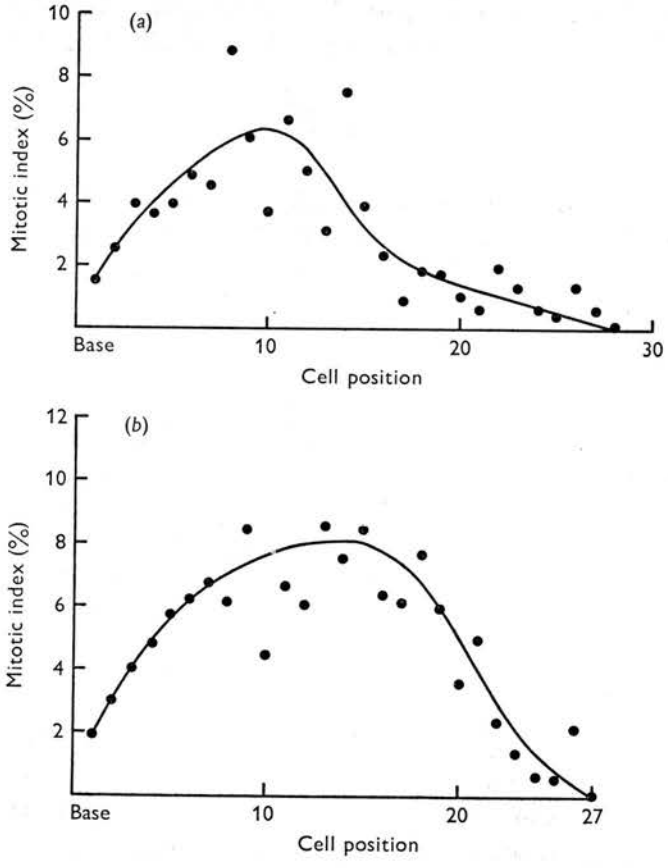

Fig. 9*a* and *b*. Distribution of mitoses in jejunal crypts of mice calculated on a cell positional basis. The upper curve (*a*) is for the castrated (control) group, and the lower curve (*b*) is for the castrated testosterone-treated animals.

value for the control and testosterone-treated group respectively. In the controls this position was 14·2, and in the testosterone-treated group, 18·7.

These results show that the proliferative compartment was increased in size by four cell positions, and imply that the position in the crypt at which cells take the critical decision to differentiate, or the 'cut-off position', is similarly raised.

The GF is also given by the ratio of the number of cells in the proliferative compartment to the total number of cells in the crypt. For the control animals this was 14·2/27·0 = 0·52, and in the testosterone-treated animals 18·7/27·0 = 0·69. This method of deriving the growth fraction by direct observation is independent of phase distribution considerations, and confirms the calculated increase (Table 2).

The MI distribution curves (Fig. 9) show essentially the same characteristics as the LI curves, but, because of the smaller indices there is greater variation between adjacent cell positions. In both groups the MI tended to fall more slowly than the LI, in accord with the crypt model proposed by Cairnie *et al.* (1965*b*).

Cell velocity

The rate of movement at any cell position is determined by the mitotic activity beneath that position. This activity can be measured by calculating the cumulative mitotic rate (MR) of all cells up to that position (Cairnie *et al.* 1965*a*). The mitotic rate of any position is

$$\mathrm{MR} = \frac{\mathrm{MI}(\%)}{t_m \times 100}. \tag{5}$$

The individual mitotic rates for each position were calculated and the cumulative MR for control and testosterone-treated animals is shown in Figs 10 and 11. For the

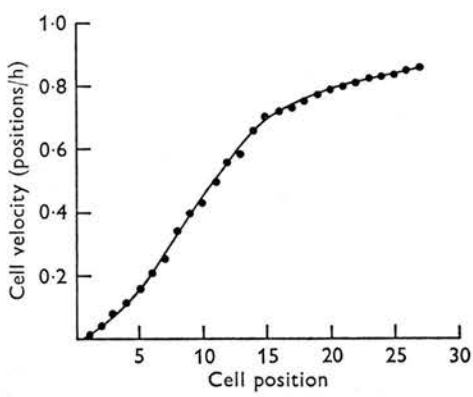

Fig. 10

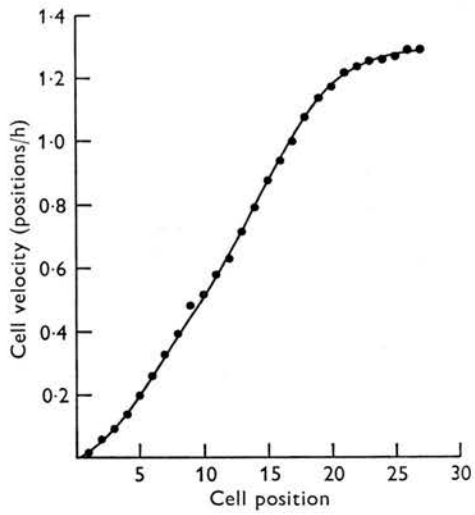

Fig. 11

Fig. 10. Cumulative mitotic rate in the jejunal crypts of castrated (control) mice. The maximum or limiting velocity was 0·8 cell positions per h.

Fig. 11. Cumulative mitotic rate in the jejunal crypts of castrated testosterone-treated mice. The maximum or limiting velocity was 1·3 cell positions per h.

reasons given above, t_m was assumed to be 1 h in each case. The maximum velocity attained was 0·8 cell positions/h in the control, and 1·3 positions/h in the testosterone-treated groups. Up to cell position 12 the rate of increase appeared to be the same in each group. The larger limiting velocity in the testosterone-treated animals is a consequence of the increased size of the proliferative compartment in this group.

An independent measurement of cell velocity can be obtained by following the movement of labelled cells up the crypt with time. The cell position of the 50 % peak was determined for all animals killed up to 4 h after administration of [^{3}H]Tdr. Labelled cells appeared on the villi soon after 4 h in both groups. The movement of the 50 % peak with time is shown in Figs 12 and 13. Lines of best fit were obtained

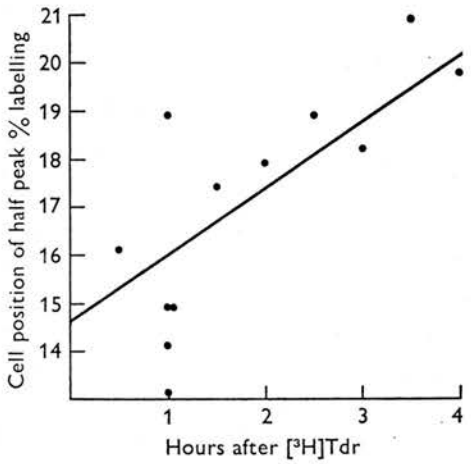

Fig. 12

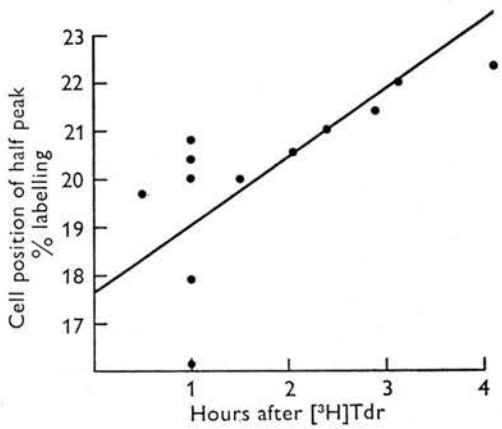

Fig. 13

Fig. 12. Movement of the position of the 50 % peak value of the labelling index distribution curve with time in the jejunal crypts of castrated (control) mice after tritiated thymidine ([^{3}H]Tdr).

Fig. 13. Movement of the position of the 50 % peak value of the labelling index distribution curve with time in the jejunal crypts of castrated testosterone-treated mice after tritiated thymidine ([^{3}H]Tdr).

by regression analysis. The slopes are a measure of cell velocity and were not significantly different. The common slope was 1·35 cell positions/h. The intercept in the control group was $14{\cdot}6 \pm 0{\cdot}8$, while that of the testosterone-treated animals was $17{\cdot}7 \pm 0{\cdot}6$. These intercept values are regarded as a measure of the position of the 50 % peak at zero time. A t-test on these values showed that the difference between them was highly significant ($P < 0{\cdot}001$).

The time at which the 50 % peak reached the top of the crypt, or foot of the villus was found by extrapolation of the regression line to the mean crypt length. This gave a value of 9·2 h for the control and 6·9 h for the testosterone-treated groups. The mean time between the uptake of [^{3}H]Tdr and the completion of mitosis is given by $0{\cdot}5t_s + t_{g2} + t_m$. This was 5·9 h for the controls and 5·3 h for the testosterone-treated animals. The difference between this value and the time taken for 50 % of the villi to become labelled reached by extrapolation of the 50 % peak value line

gives the transit time through the maturation compartment (Cairnie *et al.* 1965*a*). This was 3·3 h in the controls and 1·6 h in the testosterone-treated animals.

An experimental value for the 50% villus labelling time (the time taken for 50% of villi to become labelled) was 12–14 h in the control and 9·6–10·7 h in the testosterone-treated animals. Using these values, the transit time through the maturation compartment became 7·1 h in the control group and 4·8 h in the testosterone-treated animals.

As mentioned above, the minimum villus labelling time was approximately 4 h in both groups. An experimental estimate of the total transit time from labelling gave values of 42 h in the control and 38 h in the testosterone-treated animals.

DISCUSSION

The measurement of the phases of the cell cycle using the labelled mitoses method is open to various criticisms (Quastler & Sherman, 1959; Wright, 1971) but is no doubt the best technique available *in vivo*. Difficulties are also encountered in the comparison of parameters between two groups. In view of the very small differences obtained between the control and experimental groups compared with the total duration of the individual phases, we conclude that no real difference exists between them. However, the shapes of the two labelled mitoses curves differ. In the testosterone-treated group three distinct cycles were obtained, indicating an extremely narrow spread of cell cycle times. The control curve shows severe damping, suggesting a greater spread in cell cycle times. Rapid randomization then prevents further peaks (Wright, 1971). It should be noted, however, that these curves are for the whole crypt and, as Cairnie *et al.* (1965*a*) have shown, there is a general lengthening in cell cycle parameters towards the top of the crypt.

Measurement of mitotic duration by the labelled mitoses method is a difficult procedure. Clarke (1970) showed that this technique usually over-estimates t_m. A calculated value as described above makes important assumptions, namely the presence of steady-state conditions and asynchrony. Procedures such as castration and testosterone treatment could well modify the steady state found in the normal small bowel. Whilst any great degree of synchrony was excluded, such calculations are sensitive to even a small degree of synchronization. Therefore a duration of 1 h was assumed in calculating the cumulative mitotic rate.

The cell cycle time in the rodent small bowel is one of the shortest found in mammalian renewal systems. In any induced stimulation of cell production there is small scope for a decrease in T_c (Cairnie *et al.* 1965*b*). Testosterone was found to significantly increase the LI above that of castrated control animals. In the absence of any decrease in T_c, there must be an increase in the proportion of crypt cells actually engaged in DNA synthesis. This means an increase in the growth fraction. Such an increase was demonstrated by two independent methods (Table 2). The calculated value assuming steady state conditions was confirmed by direct observation, which, as we have noted, is independent of phase distribution considerations. Cairnie *et al.* (1965*b*), in formulating a kinetic model of crypt dynamics, concluded that a 'slow cut-off model' most appropriately fitted the experimental findings. The cell position of the 50% peak is therefore only an indication of the area over

which the transition from proliferative to non-proliferative cells occurs. The movement upwards of the position of the 50 % peak implies a similar movement of the cut-off position. This results in an increase in the size of the proliferative compartment, and therefore of the growth fraction.

The cell velocity as measured by the cumulative mitotic rate does not agree well with the velocity measured by the movement of the 50 % peak. Furthermore, the transit time through the maturation compartment obtained by extrapolation of the regression lines in Figs. 7 and 8 is less than the experimental estimate. These differences can be partly explained by difficulties in the measurement of the 50 % peak value from samples of 30 crypts from only one animal where considerable variation is found between the LI at adjacent cell positions. The period over which the 50 % peak is observed is short, since after 4 h labelled cells begin to appear on the villi. Using this method a significant difference in cell velocities between the control and the testosterone-treated animals was not demonstrated. The value for the 50 % villus labelling time found by extrapolation was 9 h in the control and 7 h in the testosterone-treated animals, a difference of 2 h, and a consequence of the increased size of the proliferative compartment in the latter group. However, the experimental 50 % villus labelling time in the testosterone-treated animals was about 3 h less than in the controls, indicating to some extent the unreliability of the cell velocity measured by movement of the 50 % peak. The total transit time from labelling was also less in the testosterone-treated mice. The maximum or limiting velocity of the cumulative mitotic rate curves would suggest faster movement in the testosterone-treated animals. Whilst free of the above criticisms, it should be remembered that an assumed value for t_m was used, and further it was assumed that no variation in t_m occurred with cell position in the crypt.

Previous studies of androgen action on small intestinal kinetics have led to widely differing conclusions. Carriere (1965) found that testosterone had no significant effect on the MI in the small bowel of the castrated male rat. Tuohimaa & Niemi (1968), however, were able to demonstrate a significant rise in the labelling index of the small bowel when testosterone was given to castrated male mice. These latter workers also found a small decrease in t_s in testosterone-treated animals, but further claimed a large reduction in t_{g1}. This caused the cell cycle time to decrease from 22 h in castrated controls to 16 h in castrated testosterone-treated animals. On the contrary our results show no significant reduction in T_c, and we believe the higher LI in the testosterone-treated animals is a reflexion of the demonstrated rise in growth fraction.

Several other hormonal situations have been shown to affect proliferative state in the small bowel. Leblond & Carriere (1955) demonstrated the important role of growth hormone in the control of small bowel kinetics, and Carriere (1965) and Wall, Middleton, Pearse & Booth (1970) have emphasized the profound effect of hypo- and hyperthyroidism. We have shown that the stimulatory effect of testosterone is mediated via the cut-off position and the growth fraction, and further work is required to find the mode of action of these other modifying factors. In this context, Cairnie & Bentley (1967) studying small bowel hyperplasia in lactation, found no increase in the size of the proliferative compartment despite a marked increase in both crypt and villus cell populations.

What is the mechanism by which testosterone affects the cut-off position? Food intake is an important parameter of small intestine proliferative state (Jervis & Levin, 1966). However, Jervis & Levin (1966) concluded that small intestine enlargement in chronic alloxan diabetes was not solely due to the increased bulk of the diet. Wierda (1950) further showed that increased food bulk was not an adequate stimulus for small intestine enlargement. Testosterone acts directly on the cut-off position to control the size of the proliferative compartment, and therefore the rate of cell differentiation in the small bowel of the castrated animal. Conceivable sites of action include the cut-off position itself or the stem cell population at the base of the crypt. Similar displacements have been found after small bowel resection (Loran & Crocker, 1963) and irradiation (Cairnie, 1967).

Testosterone also increased the growth fraction in the target accessory sex glands of the castrated animal (A. R. Morley & N. A. Wright, in preparation). Consequently this effect on growth fraction could be a general effect on both target and non-target tissues in the castrated animal. In this context, Epifanova (1966) showed that oestrogen caused a significant increase in the growth fraction of the uterine epithelium of castrated mice. There was, however, no increase in growth fraction in a non-target tissue, the cornea. In this respect testosterone may differ in its mitogenic action on non-target tissues.

We thank Professor A. G. Heppleston for continued encouragement and advice. Skilled technical assistance was rendered by Mrs E. Moody and Mr and Mrs H. Elliott. This work was performed while one of us (N.A.W.) was in receipt of a Board of Governors Cancer Research Grant from the United Newcastle upon Tyne Hospitals.

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Virchows Arch. B Cell Path. 34, 299–309 (1980)

Virchows Archiv B
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The Effect of a Single Injection of Cytosine Arabinoside on Cell Population Kinetics in the Mouse Jejunal Crypt

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Summary. The early effects of a single injection of cytosine arabinoside (ara-C) on cell population kinetics in the jejunal crypt of the mouse were studied using autoradiography with tritiated thymidine, and metaphase arrest with vincristine. Ara-C had three main effects on crypt cells: a block of cells near the transition from G_1 to S, death of nearly all cells in S, and a temporary block of the survivors, which remained viable and were able to proceed through the cell cycle. Throughout the crypt there was a decrease in cell cycle time and an increase in growth fraction. Although changes in proliferative rate were highest in the lowest part of the crypt it was not possible to show that crypt repopulation originated only from basal crypt cells, and the data are consistent with repopulation from the faster cycling cells in the proliferative compartment.

Key words: Cytosine arabinoside – Cell proliferation – Intestine.

Introduction

Opinions differ concerning the number and proliferative status of clonogenic cells in the small intestinal mucosa. Several workers concerned with the measurement of crypt survival after irradiation have suggested that all proliferative cells in the small intestinal crypt are clonogenic, and in the mouse number about 150 (Withers and Elkind 1971; Hagemann, Sigdestad and Lesher 1972; Cairnie 1976). However, Potten and Hendry (1975) have reported, on the basis of split-dose irradiation experiments, that in the mouse there are only about 80 clonogenic cells.

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0340-6075/80/0034/0299/$02.20

As to the proliferative status of these cells, Potten et al. (1976, 1977) has suggested that the clonogenic population is largely made up of cells confined to the crypt base, cycling slowly compared with cells in the remainder of the proliferative compartment. He and his colleagues consider that these cells are important in crypt homeostasis, being responsible not only for repopulation after radiation and cytotoxic damage, but also for the circadian rhythms in proliferative indices.

There is little doubt that basal crypt cells are cycling slowly, both in the rat (Carnie, Lamerton and Steel 1965; Wright, Morley and Appleton 1972; Al-Dewachi et al. 1974) and in the mouse (Al-Dewachi et al. 1975, 1978); the results indicate that in both species the bottom 8 proliferating cells have longer mean cycle times than cells in higher positions. Lamerton (1972) has pointed out the consequences of the existence of such a subpopulation: they would be less likely than rapidly dividing cells to be in a sensitive phase when exposed to irradiation or cytotoxic chemotherapy, and would thus be available for crypt repopulation. In this context Al-Dewachi et al. (1977) have reported that basal crypt cells are the first to respond kinetically after hydroxyurea-induced damage in the rat, with large increases in labelling, index due to a shortened cell cycle time.

Long cycling basal crypt cells are now regarded as 'functional' stem cells for the proliferative compartment (Cheng and Leblond 1974; Cairnie 1976); that is, they supply cells to the rest of the crypt in normal circumstances. It is an attractive hypothesis that these cells are also 'potential' stem cells which repopulate the crypt after death of proliferative cells. However Boarder and Blackett (1976) have reported that a single injection of cytosine arabinoside (ara-C) kills some 80% of clonogenic cells. Such a finding is inconsistent with any major portion of the clonogenic cell compartment being long cycling.

It thus becomes important to investigate the kinetic response of the mouse crypt to a single injection of ara-C and to assess the kinetic role of basal crypt cells in repopulation.

Materials and Methods

Male Balb/c mice aged 3 months, weighing 25–30 g were used throughout. They received 400 mg/kg body weight of ara-C (1-β-D-arabinofuranosylcytosine, Cytarabine, Upjohn Ltd.,) by intraperitoneal injection at 09.00 h. In the labelling experiments mice received 1 μCi/g body weight of tritiated thymidine (Radiochemical Centre, Amersham, England). The animals were killed by cervical dislocation, samples of small intestine were removed at a site immediately distal to the ligament of Treitz, and fixed in Carnoy's fluid for 6 h. Paraffin sections were cut a thickness of 3 μm and, where appropriate, autoradiographs were prepared as previously described (Al-Dewachi et al. 1974). Sections for counting were stained by Harris's haematoxylin, and for histological studies by haematoxylin and eosin.

Experiment 1

Thirty-two mice were each given a single injection of ara-C, and one animal was killed every hour during the first twenty-four hours, then every 3 h until 48 h after injection. Animals received

tritiated thymidine (^{3}H-TdR) 1 h before death. One hundred axially sectioned crypts were selected as previously described (Wright et al. 1972); cells were numbered 1, 2, 3, ..., n from the base to the crypt-villus junction, and the labelling and mitotic index calculated for each cell position. The data allowed construction of labelling and mitotic index distribution curves in which the labelling or mitotic index was plotted as a function of cell position in the crypt. Changes in the crypt population were monitored by counting the number of cells in the crypt column and, in transverse sections, the number of columns.

Experiment 2

A fraction of labelled mitoses (FLM) experiment was carried out starting 15 h after ara-C administration; at this time 38 animals were given ^{3}H-TdR, and one was killed every 30 min for 12 h, then every hour until 26 h after ^{3}H-TdR administration. Axially sectioned crypts were selected, and the "left-hand" column of cells was divided into nine segments counting from the bottom comprising cell positions 1 and 2, 3 and 4, ..., 15 and 16, and 17 and above. Counts of labelled and unlabelled metaphases and anaphases were made as previously described (Al-Dewachi et al. 1974), and the resulting FLM curves were analysed by the method of Gilbert (1972).

Experiment 3

A stathmokinetic experiment was carried out starting 15 h after ara-C administration. Animals were given 1 mg/kg body weight of vincristine sulphate (Oncovin, Lilly) by intraperitoneal injection and killed serially, one very 15 min until 2.5 h after vincristine. In each animal the positions in the crypt column of mitotic nuclei were noted in 100 axially sectioned crypts, and the data were analysed as previously described (Wright et al. 1972).

Results

Histology

Degenerative changes following ara-C administration were observed in the cells of the crypts of Lieberkühn only; the villous epithelium showed no signs of damage. The degenerative changes were confined to the cells of the proliferative zone; maturing cells in the upper third of the crypt, and cells in the lowest positions, were unaffected. Damage was first noted after 2 h and was severest at 5 h. Thereafter the proportion of affected cells steadily diminished, and by 20 or 21 h the crypts were almost clear of damaged cells.

Complete inhibition of thymidine uptake took place within an hour of ara-C administration, and no damaged cells were labelled.

Morphometry

Nine hours after ara-C the mean number of cells per crypt column had decreased from a control value of 25.1 ± 0.2 (S.E.) to 21.1 ± 0.2 cells (see Fig. 1). The column count also showed a small but significant reduction. Thereafter the crypt column length showed an irregular increase towards normal values, but control levels were not reached by the end of the experimental period 48 h after ara-C. The column count, on the other hand, recovered quickly after its minimum at 7 h, and by about 16 h values were close to normal.

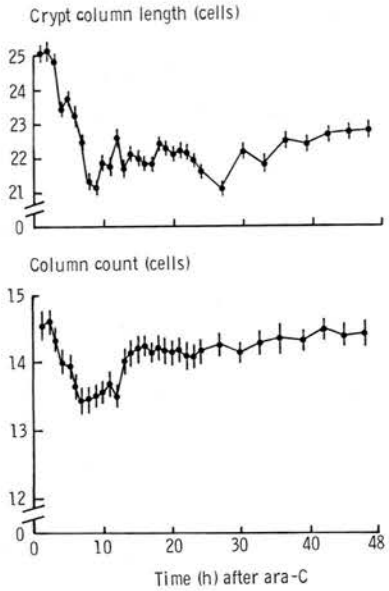

Fig. 1. The changes in crypt column length and column count in the mouse jejunal crypt after a single dose of cytosine arabinoside. The values shown are mean values with SE. Each reading indicates one animal

Labelling and Mitotic Indices

The number of labelled cells was reduced to zero during the first hour after ara-C injection (Fig. 2), while the mitotic index, although showing a significant reduction at 1 h, reached zero only after 3 h. This difference presumably reflects the time taken for cells already in G_2 and mitosis at the time of ara-C injection to complete mitosis; $t_{G_2}+t_M$ is about 2 h in Balb/c mouse crypt cells (Al-Dewachi et al. 1975).

The labelling index began to rise at 7 h and reached a peak of over 50% at 14 h, which is well above control values. There was then a fall to control levels which was maintained until the end of the experiment at 48 h. Mitotic activity also resumed at 7 h, but only a small rise in the mitotic index was evident over the period 8 to 12 h after ara-C. There was then a sharp rise to a peak of 12% at 18 h, again well above control levels; however, control values were restored by 48 h.

Distribution of Labelled Cells in the Crypt

Some distributions of labelled cells in the standardised crypt are shown for the period 7 to 21 h after ara-C in Fig. 3. At 7 h, when analysis first became possible, low labelling indices (I_s) were found at each cell position. The peak value within the proliferative compartment was around 40%, compared with a normal value of 60%. However, over the next 7 h there was a general increase in I_s at all cell positions, and at 14 h the value in the proliferative compartment was about 80%.

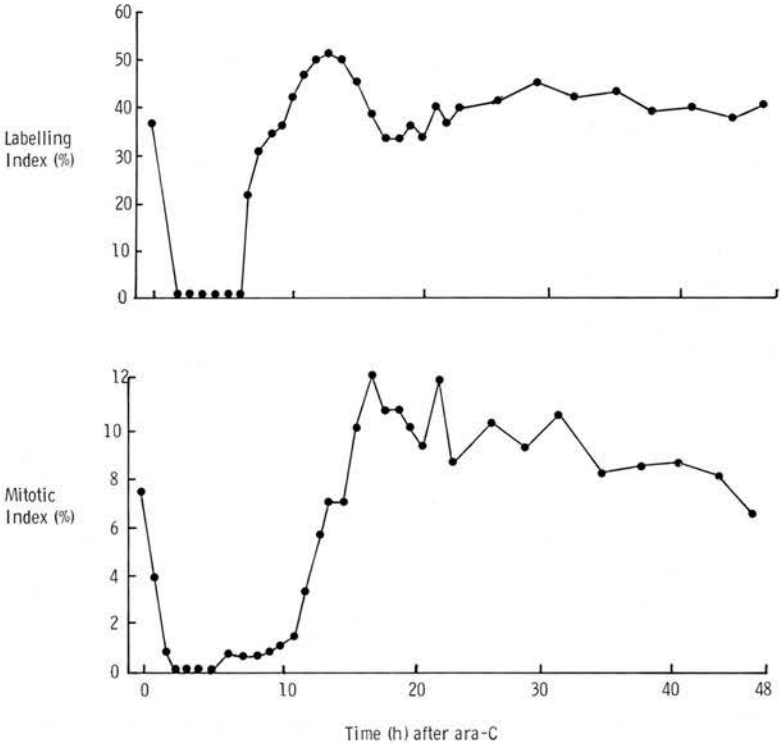

Fig. 2. Labelling and mitotic indices in the mouse jejunal crypt after a single injection of cytosine arabinoside

The site of the presumptive stem cell compartment in the crypt is of particular interest (Potten et al. 1977); 7 h after ara-C I_s values are especially low, and only show increases as part of the general response of all proliferating crypt cells. The early disproportionate increase in labelling indices in basal cell positions, found in the rat crypt after hydroxyurea (Al-Dewachi et al. 1977) is not reproduced. After ara-C the basal crypt cells do not appear to be the only cells responsible for repopulating the crypt, and the labelling index data suggest that repopulation is occurring throughout the proliferative compartment. However, it should be noted that the I_s in the lowest cell positions rises proportionately much more than in cells in the rest of the crypt, between 7 and 14 h after ara-C.

FLM Data

The FLM curves for the whole crypt in control and ara-C treated mice begun 15 h after injection are shown in Fig. 4. Table 1 shows the mean durations of the cell cycle phases for ara-C treated mice and the mean values for the

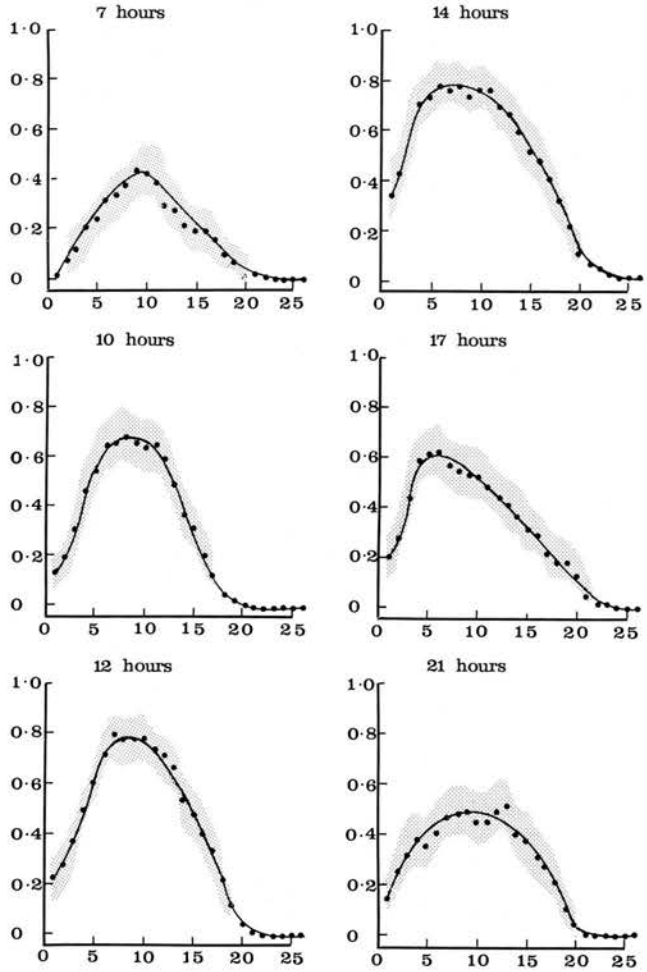

Fig. 3. A selection of labelling index distribution curves in jejunal crypts of mice at the times indicated after a single injection of cytosine arabinoside. The shaded areas indicate 95% confidence limits for the points

cell cycle time (T_c) at different cell positions in control mice (Al-Dewachi et al. 1978).

The mean cell cycle time for the whole crypt is 9.9 h compared with a control value of 12.3 h. This change is brought about mainly by a decrease in t_{G_1}, but a small change in t_s also contributes.

The greatest decreases are in cell positions below 10, and are most pronounced in positions 3 to 8. These are brought about mainly by changes in t_{G_1} and t_s; t_{G_2} remains close to control values. We are aware that cell migration makes the actual values for the phase times at any particular position uncertain, but comparisons may be valid between treated and control animals, of course assuming that migration rates are not markedly different.

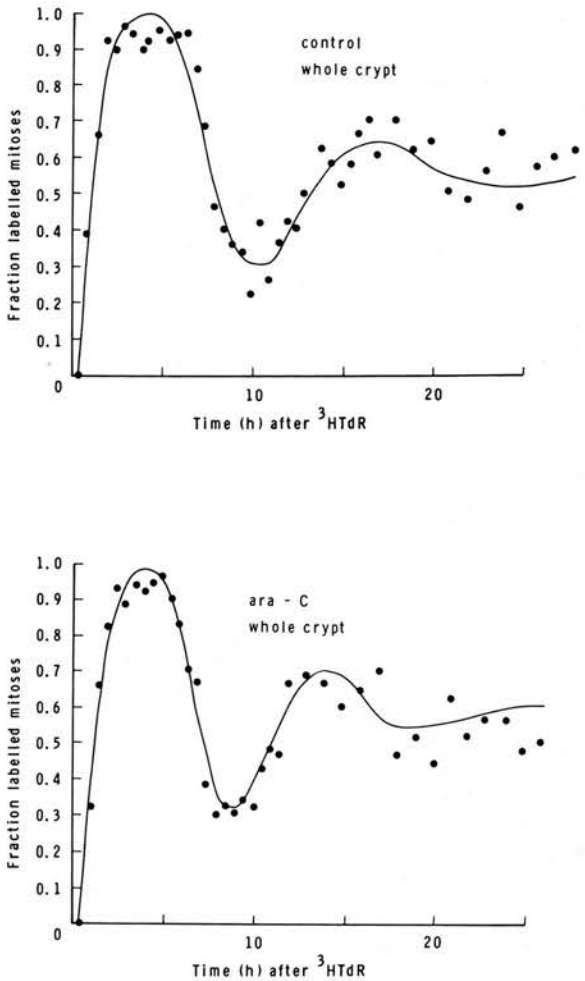

Fig. 4. Fraction of labelled mitoses curves for the whole crypt of control mice, and of mice treated with cytosine arabinoside. Tritiated thymidine was injected at fifteen hours after ara-C. The points are the experimental values, while the lines are fitted by the Gilbert program

Table 1

Cell positions	$t_{G_1}+{}^1/_2$ m	t_s	$t_{G_2}+{}^1/_2$ m	T_c	CV(T_c) (%)	Controls T_c
1–2	4.3	7.3	2.5	14.1	22	16.7
3–4	4.3	5.8	1.3	11.4	29	14.9
5–6	2.2	6.1	1.8	10.1	25	13.6
7–8	2.4	5.9	1.7	10.0	24	13.3
9–10	3.1	5.8	1.3	10.2	19	12.4
11–12	2.5	6.0	1.3	9.8	24	10.9
13–14	2.8	5.3	1.2	9.2	20	10.6
15–16	2.2	5.9	1.0	9.1	22	9.7
17+	3.0	4.9	1.2	9.1	24	10.3
Whole crypt	2.7	5.7	1.5	9.9	26	12.3

Mean values (h) of the phases of the cell cycle in different parts of the jejunal crypts of male Balb/c mice according to FLM experiments begun 15 h after administration of ara-C. The standard errors given by the Gilbert method of fitting the data were approximately 0.2 for $t_{G_1}+{}^1/_2$ m, 0.15 for t_s, 0.1 for $t_{G_2}+{}^1/_2$ m and 0.25 for T_C

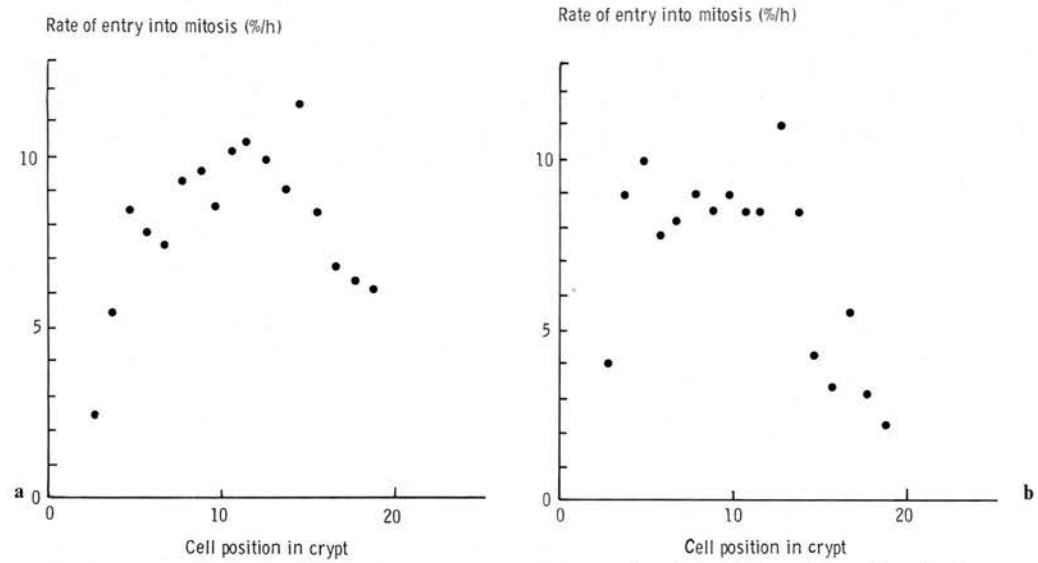

Fig. 5a and b. The rate of entry into mitosis plotted as a function of the cell position in the crypt for (**a**) control mice, and (**b**) mice 15 h after ara-C treatment

Stathmokinetic Results

The mitotic index accumulation line was fitted assuming a rectangular age distribution.

It should be noted that the mitotic index itself is increasing over this period (see Fig. 2), but in spite of this, the mitotic index rose linearly after vincristine. The rate of entry into mitosis is 5.6% of cells per hour, compared with a control value of 5.1% of cells per hour (Al-Dewachi et al. 1975). These values incorporate the factor introduced by Tannock (1967) to compensate for overestimates due to the displacement of mitoses towards the axis of the crypt.

Mitotic accumulation graphs were drawn for each cell position, (assuming an exponential age distribution at each position), and the rate of entry into mitosis (% cells/hour) was measured; these are shown, together with results from a control experiment, in Fig. 5. There are changes compared with controls; it can be seen that the rate of entry into mitosis in lower cell positions is generally increased whereas in higher cell positions (i.e., cell position 11 and above) values are either at or below control values.

Discussion

Mechanism of Action of Cytosine Arabinoside

The measurement of the crypt length and column count show that the dose of ara-C given produces cell death and subsequent cell loss in the proliferative

compartment. The labelling index was zero 1 h after ara-C injection (Fig. 2), and this inhibition of DNA synthesis lasted for 6 h. It is evident that all cells in DNA synthesis at the time of treatment were affected since the mitotic index also fell to zero in a time approximately equal to $t_{G_2}+t_m$; if cells at the G_1 to S boundary or in early S were the only ones affected, then the mitotic index would fall in a time equal to $t_s+t_{G_2}$. The rise in labelling index to a peak which exceeds control values at 14 h probably indicates that there is a degree of synchronisation, and that cells have been blocked at some point from which they have been later released to synthesise DNA. The mitotic index begins to rise noticeably at about 12 or 13 h after ara-C, which is about 5 or 6 h after the labelling index. This suggests that cells have been blocked at the transition from G_1 to S, and that this cohort then progresses through S and G_2 and into mitosis, thus accounting for the timing of the waves of labelling and mitotic indices. Graham and Whitmore (1970) concluded that ara-C killed L cells in the S phase, and caused G_1 cells to accumulate at the transition from G_1 to S; when ara-C was removed the accumulated cells proceeded synchronously through the cell cycle.

However, inspection of Fig. 2 shows an anomaly. The mitotic index is slightly greater than zero from as early as 7 h after ara-C, although it only begins a rapid increase at 12 or 13 h. This suggests that some cells (between 5 and 8% of those in S, and at all stages of S) are not killed but blocked, and with the release of the block resume DNA synthesis and proceed to mitosis. Thus ara-C would appear to have three effects on intestinal crypt cells: blocking cells at or near the beginning of the S-phase, killing most of the cells in S, and blocking but not killing others.

Proliferative Responses in the Crypt

Table 1 shows that the mean cell cycle time in the jejunal crypt of the normal mouse shortens with ascending cell position in the crypt. After ara-C treatment, T_c for the whole crypt is decreased (Table 1), and the portion of the crypt with the largest differences is the lowest 10 cell positions, notably positions 3 to 8; although, of course, it is difficult to be definitive, given the problems of statistical comparisons of cell cycle parameters obtained from FLM curves (Al-Dewachi et al. 1974). Treating rats with hydroxyurea also produces greatest reductions in T_c there, and involves the lower cell positions in a preferential increase in labelling index (Al-Dewachi et al. 1977). In the ara-C treated mouse the labelling indices at all cell positions in the proliferative compartment rise together; the absolute increases are roughly similar, so that as with hydroxyurea, the proportional increases at the bottom of the crypt are greater. The cell position at which the labelling index is half of the maximum does not change appreciably with time.

Thus we are unable to draw any firm conclusions regarding the portion of the crypt which is responsible for repopulation, beyond saying that it was not possible to demonstrate any selectively increased proliferation in basally situated cells comparable to that after hydroxyurea. The data of Boarder and

Blackett (1976), who measured fractional clonogenic cell survival after ara-C treatment, indicate that a large proportion of clonogenic cells cycle relatively quickly and are hence housed within the proliferative compartment. Our labelling index data are consistent with this proposal, with the proviso that the largest increases in proliferative rate, as assessed by the FLM and stathmokinetic techniques, were in the lowest 10 cell positions.

The sequence of changes in the labelling and mitotic index data after ara-C are interesting in the light of the finding (Boarder and Blackett 1976) that the effect on the small intestine of whole body irradiation is reduced by a preliminary injection of 200 mg/kg of ara-C 12 h before. After ara-C injection the labelling index data (Fig. 2) show that maximum synchronisation in *S* is found between 12 and 15 h; consequently the changes in radio-sensitivity after ara-C could be related to the large number of cells in the radioresistant *S* phase, as occurs in mouse jejunum after synchronisation by a series of injections of hydroxyurea (Withers 1975).

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Received June 24, 1980 / Accepted September 8, 1980

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Acta histochemica, Suppl.-Band XXVII, S. 185–194 (1983)

Zellproliferation im Magen-Darm-Kanal

Autoradiographic investigations of cell proliferation in the small and large bowel of the mouse, and the jejunal response to some abnormal conditions

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With 8 figures and 4 tables

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Introduction

Since most of the mature functional in the intestinal tract have a lifespan of only a few days, maintenance of the normal size of the functional population depends upon their constant replenishment from a delicately balanced population of proliferating cells within the intestinal crypts (Rijke 1980). Influenced by the pericryptal fibroblastic sheath (Neal and Potten 1981) and by the orientation of the muscularis mucosae (Bjerknes and Cheng 1980) the intestinal crypt forms a flexible and highly adaptable unit capable of attaining stability in many different kinetic configurations (Sunter et al. 1979).

Some of the ways in which intestinal crypt cell proliferative activity may be altered have recently been reviewed (Allpeton et al. 1980): these include the effects of hormones, cyclic nucleotides, chalones, surgical manipulations, cytotoxic agents and carcinogens. These induced changes can give us some insight into growth control mechanism in the intestinal epithelium, as also may the differences which exist between species and even between different strains in the same species.

There are many ways to study cell proliferation in the intestine: the work already cited (Appleton et al. 1980) deals with simple morphology, stereology, stathmokinetic methods and split-dose irradiation experiments, among others, and autoradiography is only one of the tools we have for our investigations. Nor is it entirely free from problems (Maurer 1981) but properly used it can produce detailed reliable data which can be readily interpreted. Preferably several techniques should be brought to bear on the same problem, and in our work we have endeavoured to complement autoradiography with other methods (Al-Dewachi 1975, de Rodriguez et al. 1979) but in this review we shall restrict ourselves to our work with tritiated thymidine (^{3}HTdR) in the mouse (Al-Dewachi et al. 1975, de Rodriguez et al. 1979, Sunter et al. 1979, Wright et al. 1972, Al-Dewachi et al. 1981).

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Material and methods

The mice used in the experiments, which have been carried out over a period of 10 years, were of 2 strains: all jejunal results refer to Balb/c mice and all colonic results to A_2G mice. Differences between jejunum and colon are so great, indeed fundamental, that they eclipse any minor variation caused by strain differences. All mice were male and aged between 12 and 16 weeks at the time of experiment. Tritiated thymidine was always given 1 h before death, at a dosage of 1 μCi per g body weight. In experiments dealing with the effects of castration this operation was carried out under ether anaesthesia at the age of 3 months. When testosterone propionate was administered it was given 14 days later in a dosage of 250 μg dissolved in sesame oil, daily for 3 days; control animals in this experiment were given similar quantities of sesame oil. In experiments using cytosine arabinoside (ara-C) this was administered in a dosage of 400 μg per g body weight.

Labelling index experiments were conducted at 9.00 h, and fraction of labelled mitoses experiments were begun at the same time, except when investigating the changes following ara-C injection.

Where we refer to *jejunum* we mean a site 1 mm distal to the ligament of Treitz; *caecum* indicates the junction of the middle and distal thirds of caeco-appendix; *ascending colon*, *transverse colon* and *descending colon* refer to positions respectively 10 %, 40 % and 70 % of the distance from the ileo-caecal valve to the anus. Blocks from these sites were processed through to paraffin wax and autoradiographs were prepared on 3 μm thick sections. Estimates of the number of cells in the crypt circumference were carriedout on crypt cross-sections; otherwise longitudinal sections were used, in which the base, middle and mouth of the crypt were all in the plane of the section.

Full details of the experimental methods, including the numbers of mice used and the numbers of crypts studied in each experiment have been published (Al-Dewachi et al. 1975, de Rodriguez et al. 1979, Sunter et al. 1979, Wright et al. 1972, Al-Dewachi et al. 1981).

We have calculated several quantities which we define as follows.

The *crypt length* is the number of cells in in longitudinal section from the base of the crypt to the crypt-villus junctions (small intestine) or the surface epithelium (large intestine). The *number of columns per crypt* is the number of cells seen in cross-section. The *total number of cells* is the product of the crypt length and the number of columns per crypt, it probably slightly overestimates the actual number of cells in a crypt. The *labelling index distribution* relates the percentage of labelled cells to cell position in the crypt; cell position 1 is at the bottom of the crypt. The *half-maximum position* (HMP) is that cell position (from the bottom of the crypt) where the labelling index falls to half the maximum value it has attained. All labelling index distributions and half-maximum positions are based on a crypt whose length is the mean of those counted in a particular experiment (Cairnie and Bentley 1957, Wright et al. 1972). From the fraction of labelled mitoses experiment we calculate the *cell cycle time* (T_C) and its component phases (Gilbert 1972); we also estimate the coefficient of variation of T_C and from the sum of this information calculate, assuming an underlying rectangular age distribution for proliferating cells (Schultz et al. 1972), the "theoretical labelling index", i.e. that which would occur if all cells were proliferating. Hence, from the ratio of the observed labelling index to the theoretical one we calculate the *growth fraction* (Appleton and Sunter 1979).

Thus we are able to quantify cell proliferation in the intestinal crypt in terms of number of cells, cell cycle time, region of proliferation and intensity of proliferation within that region.

Results

The normal mouse

Table 1 shows the data on the number of cells in the crypts at specified sites in the intestine of the normal mouse. The ascending colon has only half the cells of the other colonic sites; the caecum maintains a high population of cells per crypt due to a large number of columns per crypt, 70 % more than in the jejunum.

Fig. 1 shows the labelling index distributions at the 5 selected sites in the normal mouse intestine. Only in the jejunum is there a pronounced reduction in labelling

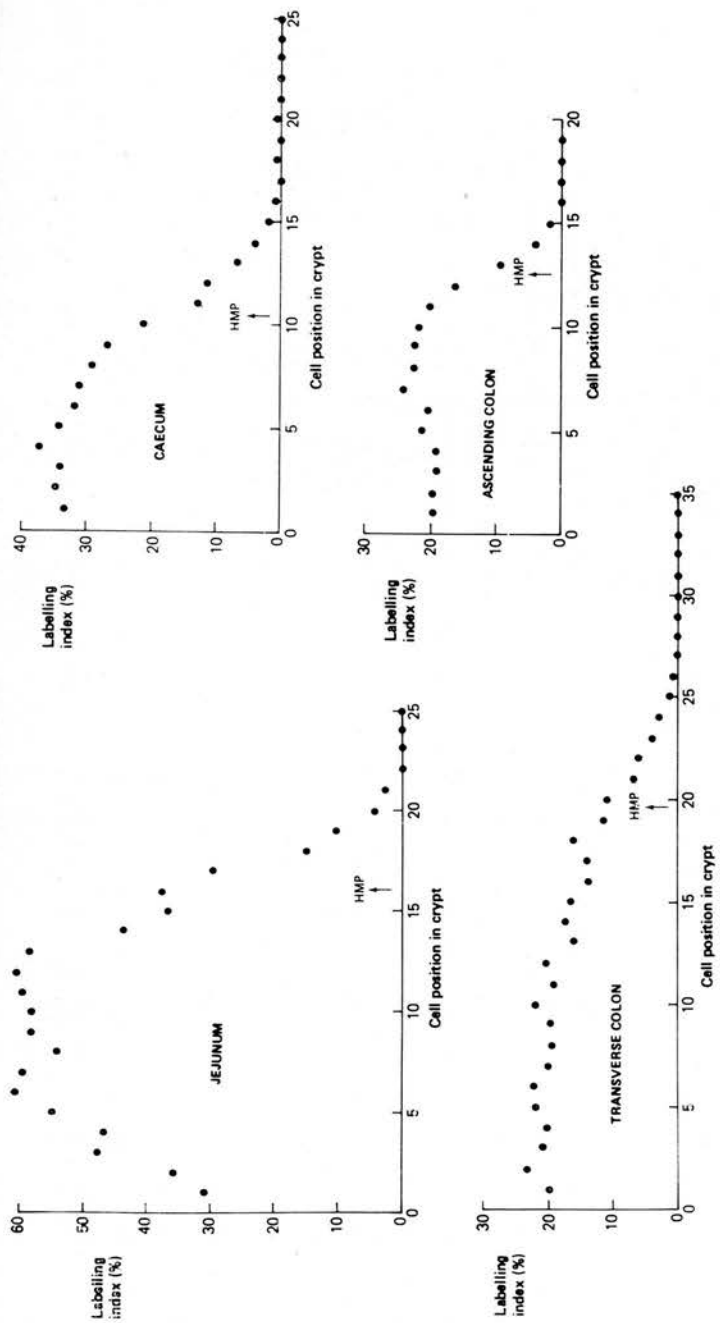

Fig. 1. Labelling index distributions in the crypt at various sites in the intestine of the normal mouse.

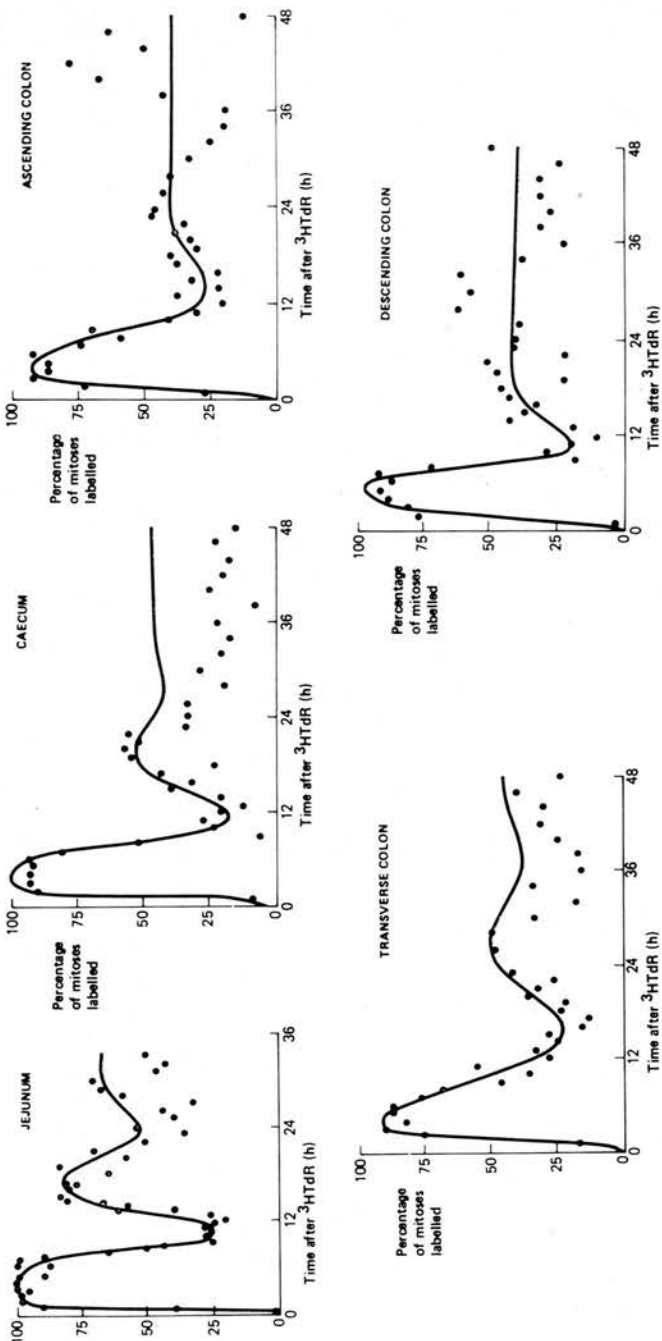

Fig. 2. Fraction of labelled mitoses data, with fitted vu ves, for the crypt at various sites in the intestine of the normal mouse.

Table 1. Data on the number of cells in the crypts at specified sites in the intestine of the normal mouse

	Jejunum	Caecum	Ascending Colon	Transverse Colon	Descending Colon
Crypt length (cells)	25.1	25.3	19.3	34.7	31.1
Number of columns per crypt	14.6	25.0	16.2	19.6	22.5
Total number of cells	370	630	310	680	700

index in basal cell positions; at no site was labelling ever seen in the topmost cell positions. Labelling index is generally lower in the colonic sites than in the jejunum; the caecum occupies an intermediate position.

Fig. 2 shows the FLM curves, fitted by the method of Gilbert, for the 5 sites. There is a well defined second wave and a good fit for the jejunal data; other sites show more variability in T_c and a poorer fit, mainly because of "fade" (Steel 1977).

The autoradiographic data (Table 2) show that T_c is least in the jejunum but does not provide any evidence of a gradient in T_c throughout the colon, though this exists in labelling index. The growth fraction in the caecum and at the 3 colonic sites is remarkably constant, about half of that in the jejunum. The half-maximum position, whether expressed as actual cell position or as a percentage of crypt length, fluctuates from site to site. None of these variables correlates with the total number of cells per crypt.

Table 2. Autoradiographic data (normal mouse)

	Jejunum	Caecum	Ascending Colon	Transverse Colon	Descending Colon
Cell cycle time (h)	12.4	15.3	18.9	21.1	15.5
Labelling index (%)	37.0	13.5	12.3	12.1	11.7
Growth fraction (%)	59	28	28	27	27
Half-maximum position (% of crypt length)	65	40	65	55	40

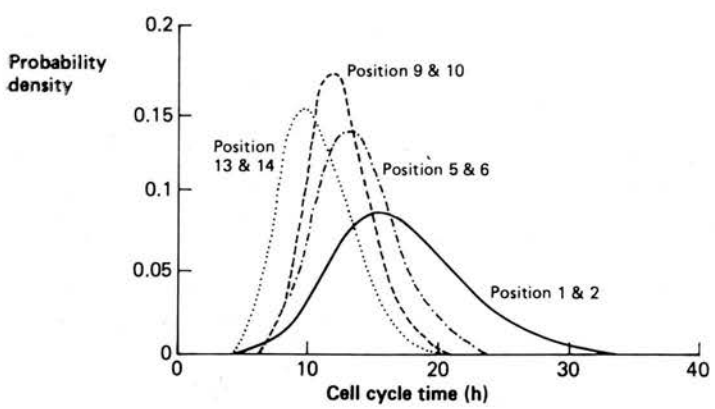

Fig. 3. Distribution of cell cycle times at various positions in the jejunal crypt of the normal mouse.

It is possible, by regarding each cell position in the crypt individually, to estimate the cell cycle time and its distribution at different parts of the crypt. This begs the question of what is meant by the cell cycle time at a particular position in a system where cells migrate through a position, but Fig. 3 shows the result of such a procedure: the mean cell cycle time in the jejunum appears to be reduced as cells progress up the crypt, and there seems to be a greater spread in cell cycle times in basal positions. This pattern is not found at the other positions in the intestine.

The jejunum of the castrate and testosterone-treated castrate mouse

Fig. 4 shows the labelling index distribution in the jejunum of castrate mice and castrates treated with testosterone. There is little change in the crypt length, but in the castrates the labelling index throughout the crypt is depressed and labelling extends less far up the crypt; in the treated mice the distribution is close to that seen in the normal jejunum.

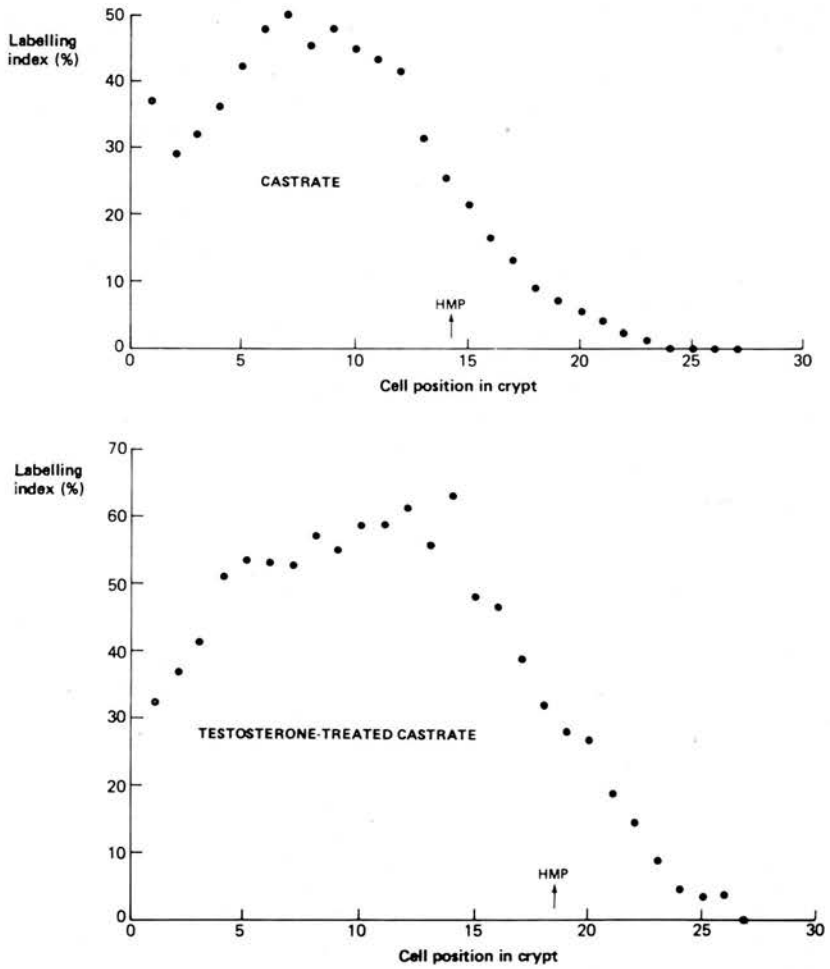

Fig. 4. Labelling index distributions in the jejunal crypt of the castrate mouse and the testosterone treated castrate mouse.

Table 3. Labelled mitoses data (mouse jejunum)

	Normal	Castrate	Testosterone-treated Castrate
Crypt length (cells)	25.1	27.0	27.4
Cell cycle time (h)	12.4	12.9	11.8
Labelling index (%)	37.0	23.4	37.4
Growth Fraction (%)	59	38	64
Half-maximum position (% of crypt length)	65	55	70

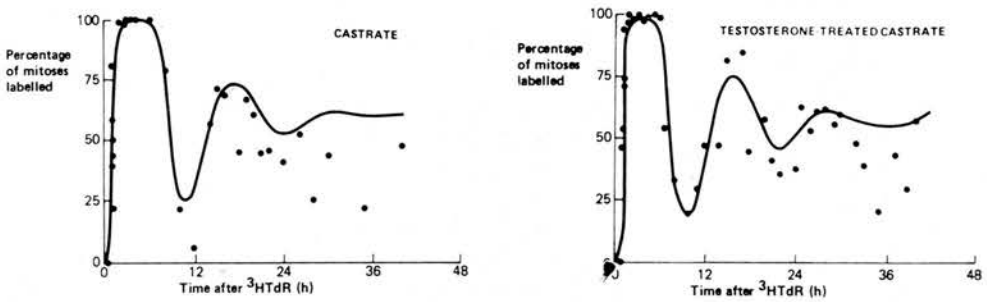

Fig. 5. Fraction of labelled mitoses data, with fitted curves, for the jejunal crypt of the castrate mouse and the testosterone-treated castrate mouse.

Fig. 5 shows the FLM data and the fitted curves. In the castrates the fit is not as good as previously, but both curves demonstrate distinct second waves indicating little variability in cell cycle times; mean T_c in both cases is close to the normal value, but there is a suggestion of a slightly reduced duration of the S phase in treated animals. The data from these experiments are summarised in Table 3.

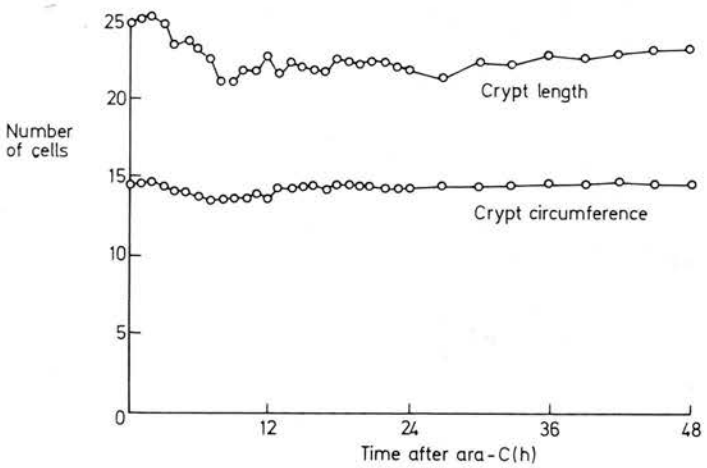

Fig. 6. The number of cells in longitudinal and transverse cross-sections (crypt length and number of columns) of he jejunal crypt of the mouse at various times after treatment with ara-C.

The jejunum of the cytosine-arabinoside-treated mouse

After treatment by are-C both the crypt length and the number of columns per crypt are reduced, as shown in Fig. 6. The change in the number of columns is small, though statistically significant, and is transient, recovery being complete by about 13 h after treatment. The crypt length, however, shows a reduction of about 4 cells (15 %) for more than 24 hours, and normal values have still not been regained by 48 hours.

Fig. 7 shows the labelling index during this time. It is reduced within an hour to zero where it remains for 6 hours; recovery is then rapid and a maximum, some

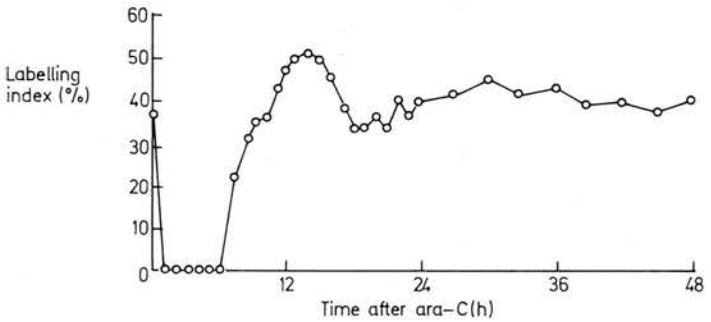

Fig. 7. The labelling index of the jejunal crypt of the mouse at various times after treatment with ara-C.

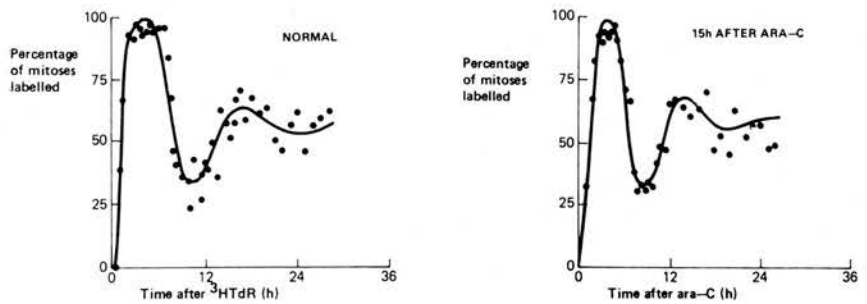

Fig. 8. Fraction of labelled mitoses data, with fitted curves, for the jejunal crypt of the normal mouse (a separate experiment from that illustrated in figure 2) and for the mouse after treatment with ara-C; for the latter experiment time 0 was 15 h after treatment.

Table 4. Labelled mitoses data (mouse jejunum)

	Normal	15 h after ara-C
Crypt ength (cells)	25.1	22.0
Cell cycle time (h)	12.4	9.9
Labelling index (%)	37.0	37.0
Growth fraction (%)	59	64
Half-maximum position (% of crypt length)	65	65

third higher than normal is attained at 13 h. A process of damped oscillations leads to a stable normal value by 48 h. There are no very great changes in the labelling index distribution during this period.

Fig. 8 compares an FLM curve begun 15 h after are-C treatment, so that the labelling index overall was close to normal values, with a control curve. Although normal jejunal cells are already cycling quickly the cell cycle time in the treated animals appears to be reduced. The data derived from this experiment are summarised in Table 4.

Discussion

The morphometric and kinetic differences between the epithelia from the jejunal and colonic sites in the normal mouse are not surprising in view of the different structure and function of the two organs, but the extent of the differences within the colon is perhaps unexpected as it is considerably greater than that found in the rat (Sunter et al. 1979). The general pattern in the two species is similar with larger crypts in descending and transverse colon than in ascending colon or caecum, but there are also considerable differences: the gradient in cell cycle times found in the rat (longer as we move distally) was not apparent in the mouse, and the distribution of DNA-synthetic cells was also quite different. For example in the descending colon of the rat there appears to be a very high proportion of cells proliferating in the bottom twelve cell positions in the crypt, as there is in the jejunum; however in the mouse the finding of a much lower estimate for the growth fraction than for the half-maximum position as a proportion of crypt length (27 % versus 40 %) suggests that fewer than 70 % of cells in what is regarded as the proliferative compartment of the crypt are in fact proliferating. This is confirmed by treating sections of the crypt separately for the purposes of an FLM study (Sunter et al. 1979). Only in the jejunum of the mouse is there a high growth fraction in the proliferative compartment.

When mice are castrated the growth fraction in the jejunal crypt drops via two separate changes: the proliferative compartment contracts and the growth fraction within it is reduced. There is, however, no reduction in the length of the crypts, nor any alteration in cell cycle time. The status quo is restored by the administration of testosterone.

On the other hand, during revovery after the administration fo ara-C, there seems to be little alteration in the distribution of proliferating cells within the jejunal crypt; instead the response is a reduction in the already short cell cycle time. This is in contrast to what happens in the rat jejunum during recovery after administration of hydroxyurea (Al-Dewachi et al. 1977). There the longer cycling cells in the base of the crypt are the principal means of recovery, but, although the cells at the base of the mouse jejunal crypt are also more slowly cycling than the cells above them, we observe no similar response to ara-C. Nor could we find evidence in the mouse colon of a change in mean cell cycle time in different parts of the crypt.

In conclusion, there are many different equilibrium states possible for intestinal epithelium under different circumstances, normal and abnormal. Crypt length and circumference may change — though in a controlled situation the crypt epithelium always remains one cell thick. Within the crypt the cell cycle time may alter, either throughout the crypt or in specific cell positions; and the growth fraction may change, through either the extent of the proliferative compartment or the proportion of cycling cells within it (see also Wright 1980). The controlling factors, whether they lie inside or outside the crypt system, remain to be elucidated, as do the relationships between the susceptibility to cancer and the proliferative characteristics.

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Virchows Arch. B Cell Path. 29, 211–223 (1978)

Virchows Archiv B
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Pathological Features of the Colonic Tumours Induced in Rats by the Administration of 1,2-Dimethylhydrazine

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Summary. The parenteral administration of 1,2-dimethylhydrazine to rats caused the development of colonic neoplasms in about 90% of animals by 24–30 weeks of treatment. Usually there were multiple tumours with a mean of 2.7 per rat. The lesions have been classified histologically into adenomata (26% of all tumours) and carcinomata, the latter showing varying degrees of differentiation. No completely anaplastic tumours were seen, and there were none originating in connective tissue. The distributions of the different tumour types along the length of the colon varied. The more benign lesions were situated predominantly in the distal half of the colon, while the poorly differentiated adenocarcinomata were concentrated in the proximal third of the colon.

There was good evidence to suggest that adenomata often progressed to frank malignancy in the distal colon. In the proximal part, however, it appeared that tumours frequently developed de novo as poorly differentiated carcinomata. Perhaps regional variations in the kinetic organisation of the normal colonic mucosa somehow influence the nature of the neoplastic change induced by DMH, thus accounting for the differences in tumor distribution.

After 24 weeks of DMH treatment there was only a small increase in the mean number of tumours per rat.

Key words: Colon – Dimethylhydrazine – Carcinoma.

Introduction

In 1967, Druckrey and coworkers reported that the parenteral administration of the chemical 1,2-dimethylhydrazine (DMH) to rats resulted in the development of neoplasms, in both small and large intestine. All treated animals were

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0340-6075/78/0029/0211/$02.60

affected and the carcinogen appeared to be selective in its effects as only intestinal neoplasms were found (Druckrey et al., 1967). Several groups of workers showed that DMH was an effective carcinogen also in mice, and in these animals it appeared that the effects were even more selective, with neoplasms occurring only in the large bowel (Wiebecke et al., 1969; Pegg and Hawks, 1971). With further experience, however, it has become apparent that the carcinogenic effects of DMH are not limited to the intestinal tract, either in rats (Druckrey, 1970; Martin et al., 1973), or in mice (Toth et al., 1976). Nevertheless, most workers agree that with appropriate dosage and treatment schedules the occurrence of colonic neoplasms both in rats and in many strains of mice can be made to approach 100%, and it is principally as a reliable colonic carcinogen that DMH has found experimental use.

Most of the colonic neoplasms seen in the rat have been adenocarcinomata, showing varying degrees of differentiation, the rest being benign adenomata (Druckrey, 1970; Martin et al., 1973; Wiebecke et al., 1973). The lesions were usually multiple and occurred throughout the length of the large intestine. While some workers have commented on a relative paucity of tumours in the proximal colon (Pozharisski et al., 1975), other groups have not demonstrated such a distribution (Martin et al., 1973; Wiebecke et al., 1973). The distribution of the different tumour types along the lenght of the bowel has been commented on: Ward (1974) stated "almost all of the mucinous adenocarcinomas were located in the ascending colon", while Pozharisski (1975) showed a high frequency of mucinous and signet ring carcinomata in ascending colon, with a preponderance of better differentiated tumours in the more distal portion of the colon. These observations are of some interest in any consideration of the mechanisms of colonic carcinogenesis by DMH, especially in view of the kinetic dissimilarities between different regions of the colon (Sunter, Wright and Appleton, 1978). And it may well be that in a given experimental situation a change in relative frequency or in distribution of a particular tumour type may have just as much significance as any change in total numbers of tumours.

The purpose of this study, therefore, is to document in some detail the histopathological types of tumour produced in the rat by DMH, and to establish their relative frequency and the distribution of the different types along the length of the colon. The changing patterns of tumour development are documented up to 30 weeks after commencement of carcinogen treatment.

Materials and Methods

Randomly bred virgin female albino Wistar Porton rats from our own colony were used throughout. The animals were between 12 and 16 weeks old when DMH injections were begun and weighed between 250 and 300 g. They were fed on standard rat cake (N.E. Farmers) and allowed tapwater ad libitum.

Symmetrical dimethylhydrazine dihydrochloride (Aldrich Chemical Co.) was administered by weekly subcutaneous injection at a dosage of 15 mg (of base)/kg body weight. The chemical was dissolved at a concentration of 1.66 g (of dihydrochloride)/100 ml of normal saline containing 1.5% EDTA added as a stabilising agent (Druckrey, 1970). The solution was brought to a pH of 6.4 by the addition of N sodium hydroxide solution; fresh solution was prepared each week.

At various times up to 30 weeks after the commencement of DMH injections animals were killed by cervical dislocation. At least one week was allowed from the final DMH injection to the time of killing in order to allow recovery from any acute toxic effects of the chemical. A full autopsy was performed on each animal, particular attention being paid to the large bowel, which was removed, opened along its length and pinned with its mucosal surface uppermost to a cork board. The whole colon was then fixed in a bath of Carnoy's fluid for six hours, and then transferred to cellosolve, in which the material remained for a minimum of a further 24 h.

For each animal the total length of the fixed colon was recorded, together with a brief description of the naked eye appearances of any tumours, including measurement of the two greatest diameters (with Vernier callipers), and site (measured in mm from the anus). Transverse blocks of all tumours were taken and processed through to paraffin wax. Histological sections 3 μm thick were prepared and stained with haematoxylin and eosin; sections from some tumours were also stained by the periodic acid-Schiff method after amylase treatment.

Results

With the regime employed acute toxic effects were not seen. In the period up to thirty weeks after commencement of treatment we found that mortality and morbidity attributable to the presence of the colonic tumours was very low. Some animals were lost, however, from the effects of malignant small-intestinal tumours, and several animals which were killed after developing ear duct or renal tumours were excluded from the analysis.

The first visible colonic neoplasms were noted after 19 weeks of DMH treatment. Over the subsequent eleven-week period till the end of the experiment material from 142 rats was accumulated, yielding 378 separate colonic neoplasms sufficiently large to be visible to the naked eye. Table 1 shows the proportion of animals affected by colonic tumours at several different times after the initiation of carcinogen treatment. At 24 weeks 92% of the rats had colonic tumours, for the smaller group at 27 and 28 weeks the proportion was 89%, and at 30 weeks all the rats were affected.

Histopathological Classification of Tumours. Macroscopically the tumours presented a variety of appearances viz. sessile or pedunculated mucosal nodules, pedunculated polyps, and more or less well defined, often partly ulcerated, mucosal thickenings or plaques. All the tumours were of epithelial origin and we have classified them as follows:-

Benign Tumours. These lesions appear macroscopically either as pedunculated polyps, or as well circumscribed, often almost spherical, mucosal nodules (Fig. 1).

Table 1. The proportion of animals affected by colonic tumours at different times after the start of DMH injections

				Totals
Duration of DMH treatment (wk)	24	27 and 28	30	24+
No. of rats killed	86	28	14	128
No. with colonic tumours	79	25	14	118
Proportion with colonic tumours	92%	89%	100%	92%

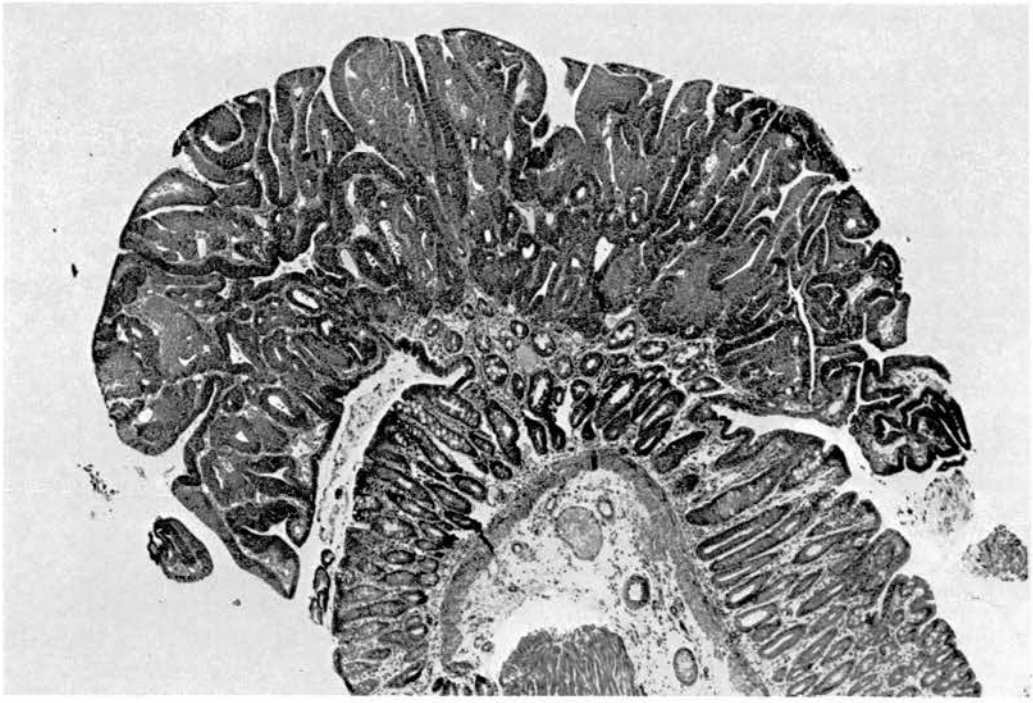

Fig. 1. Pedunculated benign adenoma. Haematoxylin and eosin, ×65

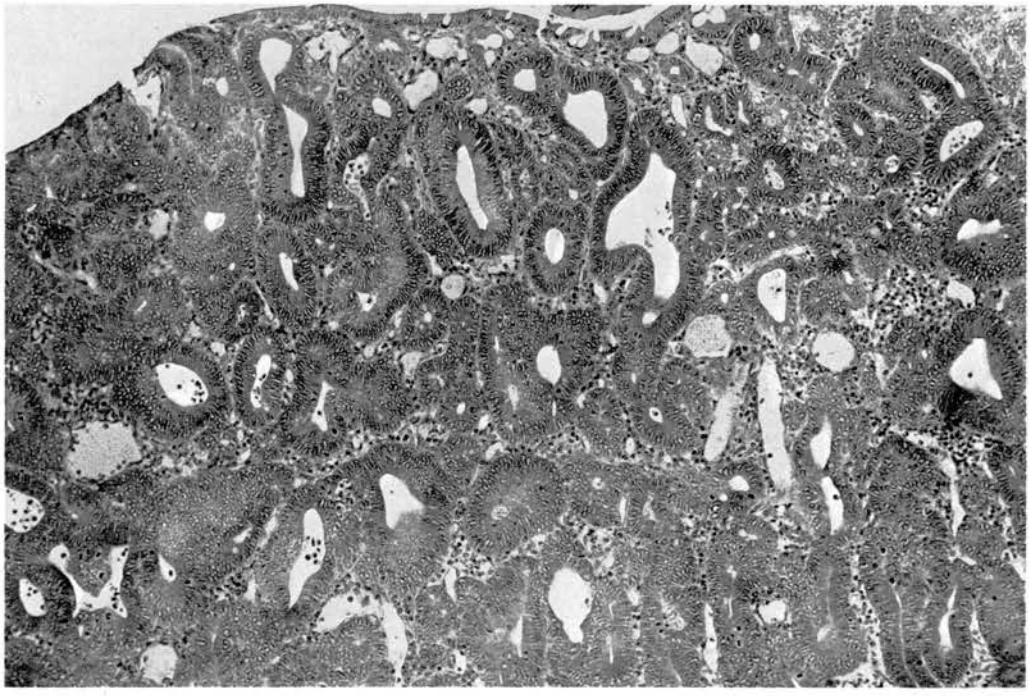

Fig. 2. Detail of a benign adenoma showing uniform tubular structure and absence of cellular atypism. Haematoxylin and eosin, ×165

Histologically the structure is that of benign adenomata, with either a purely tubular structure (Fig. 2) or a tubulovillous pattern (Fig. 3). These lesions bear a striking resemblance to the adenomata seen in the human colon though there are no instances of purely villous adenomata. The lesions are uniformly well differentiated and there is no cellular atypism to suggest in situ malignancy; ulceration is not a feature and there is no evidence of invasion through the muscularis mucosae. Ninety-eight tumours (26%) fell into this group.

Group 1 Carcinomata. The macroscopic features of this group of tumours are the same as those described above and they are histologically indistinguishable, save in one important respect: they show localised invasion into and through the muscularis mucosae (Fig. 4). This invasive component is less well differentiated and represents a frank expression of malignancy. We regard these tumours as a distinctive group of "early" invasive, well-differentiated adenocarcinomata. A total of 61 tumours fell into this group, comprising 16% of all tumours and 22% of carcinomata.

Group 2 Carcinomata. Macroscopically these tumours are mucosal nodules or well circumscribed plaques; often, invasion of deeper structures in the bowel wall is obvious. Histologically the appearances are those of frankly invasive adenocarcinoma, well differentiated or moderately well differentiated, with extensive invasion of the bowel wall a frequent feature. Surface ulceration is often conspicuous and secondary inflammatory changes are common (Fig. 5). Many, but not all, of these tumours show at their periphery residual adenomatous tumour tissue (Fig. 6), suggesting origin from a better differentiated antecedent neoplasm. One hundred and fifty two tumours of this type occurred, comprising 40% of all tumours and 54% of carcinomata.

Group 3 Carcinomata. The largest tumours fall into this category, individual tumours measuring some 15 mm in diameter. Grossly they appear either as nodules or as curious fusiform plaques, and surface ulceration is usual. Microscopically the appearances are those of poorly differentiated mucin-secreting adenocarcinoma, frequently invading through the full thickness of the bowel wall. The usual structure is that of closely packed, poorly formed acini, but there are areas of "signet ring carcinomas" (Fig. 7) and areas of so-called colloid carcinoma consisting of irregular neoplastic acini lying in pools of mucus (Fig. 8). In the majority of these tumours there is no evidence of any antecedent benign growth. This group included 67 tumours, comprising 18% of all tumours, and 24% of malignant ones.

Completely anaplastic tumours have not been seen, nor were there any non-epithelial primary tumours in the colon. Table 2 summarises the relative frequencies of the different classes of tumour. Benign tumours account for about one quarter overall, while the rest of the lesions are some form of carcinoma.

Tumour Distribution. The location of each individual colonic tumour was expressed as a percentage of the total colonic length, measured from anus to ileocaecal valve. Figure 9 shows the distribution of all tumours. Examination

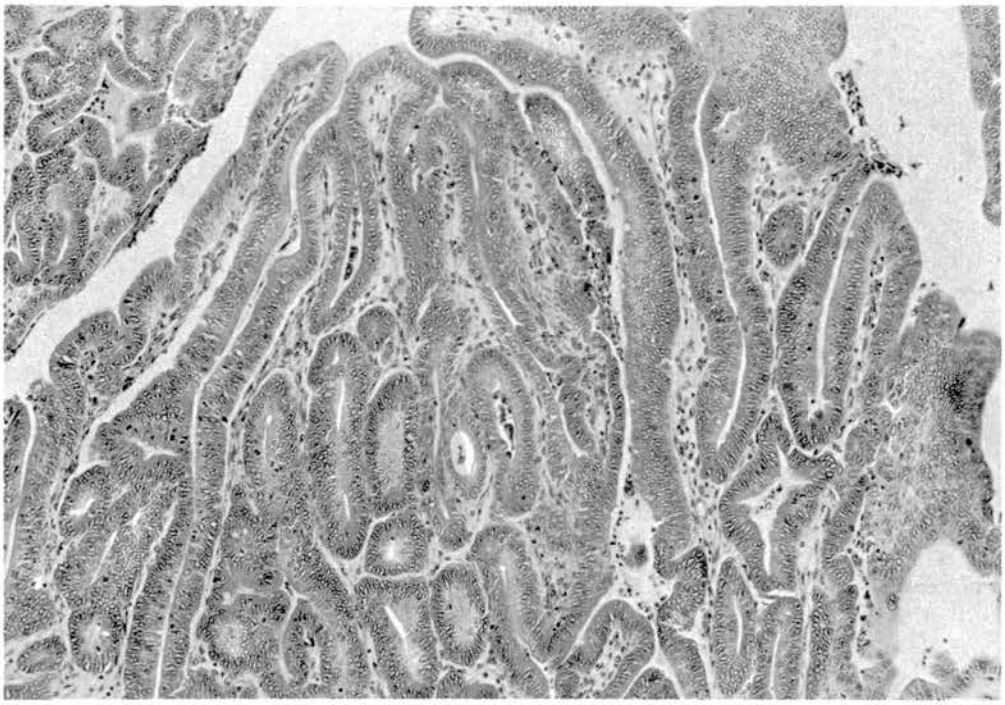

Fig. 3. Detail of a benign adenoma showing a tubulovillous pattern. Haematoxylin and eosin, ×165

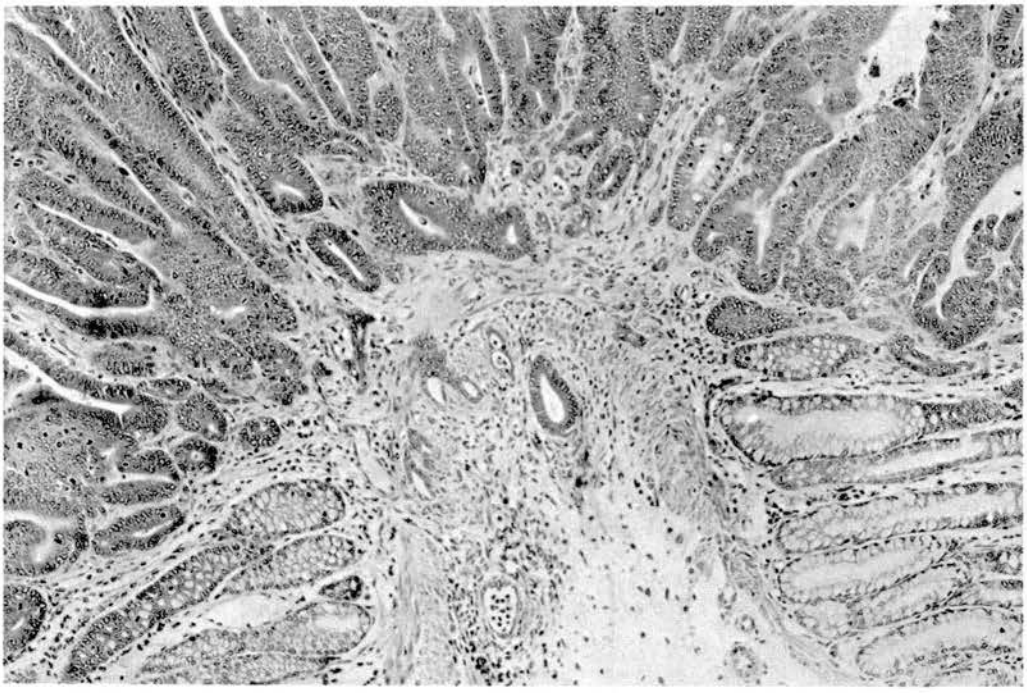

Fig. 4. Invasion through the muscularis mucosae in a group 1 carcinoma. The associated inflammatory reaction with "cryptitis" is a common feature. Haematoxylin and eosin, ×165

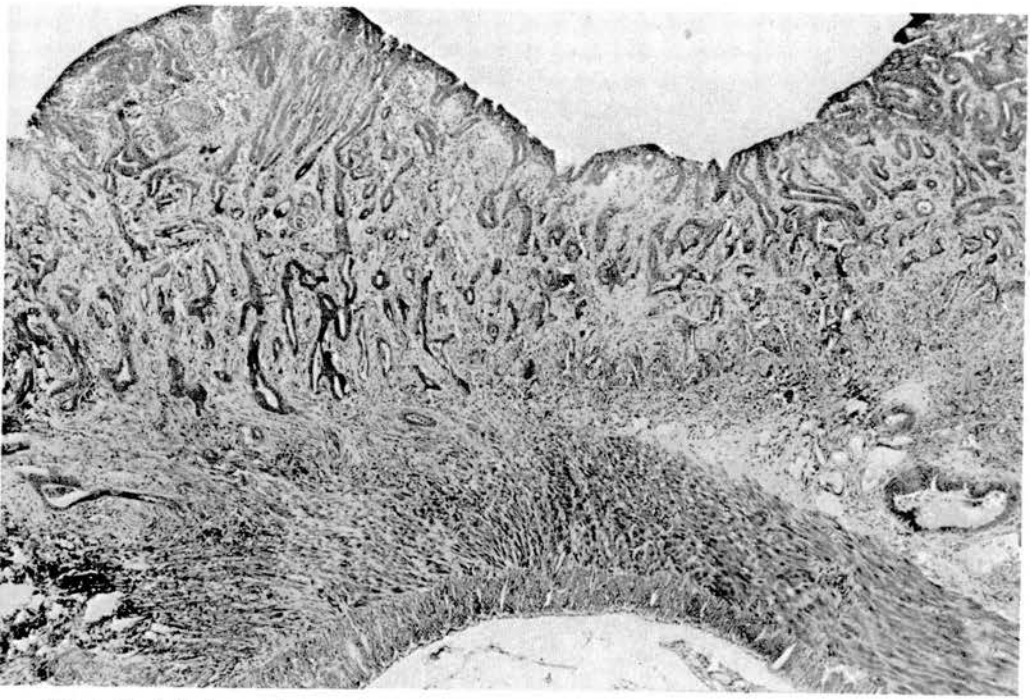

Fig. 5. Typical ulcerated group 2 carcinoma, showing a conspicuous fibrous stromal reaction. Haematoxylin and eosin, ×65

Fig. 6. The edge of a group 2 lesion showing a peripheral zone of well differentiated tumour. Haematoxylin and eosin, ×165

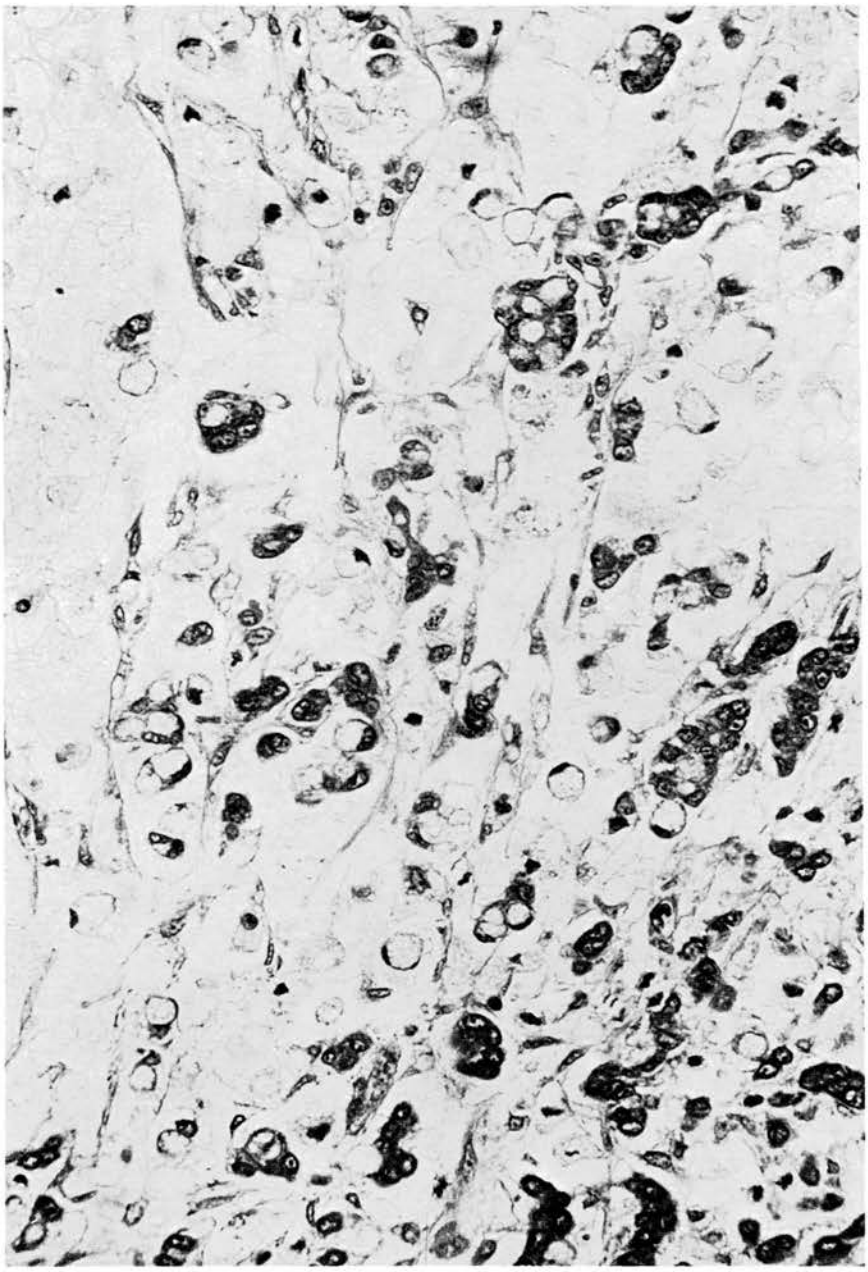

Fig. 7. A group 3, poorly differentiated, adenocarcinoma, showing signet-ring cells. Haematoxylin and eosin, ×410

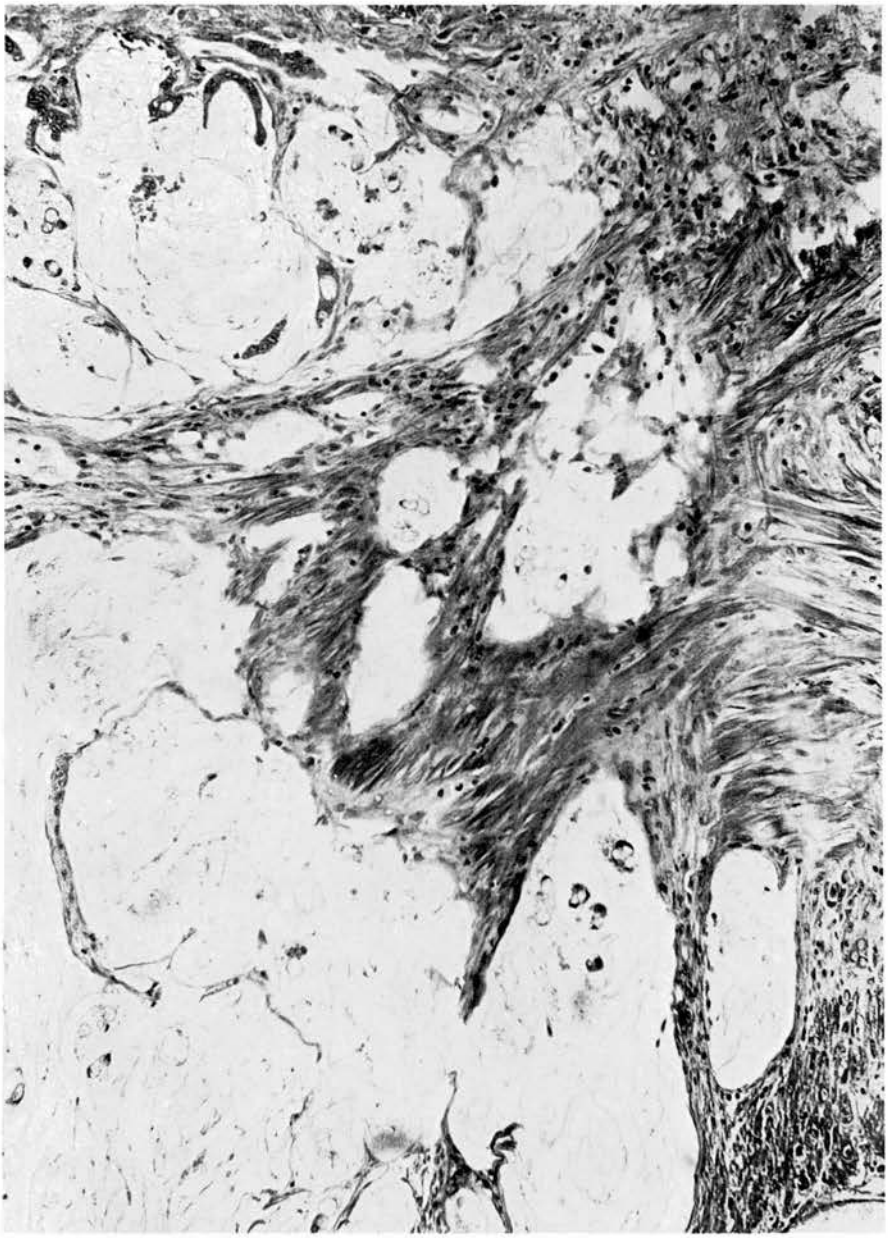

Fig. 8. A group 3 carcinoma invading muscularis propria showing "colloid carcinoma" appearances. Haematoxylin and eosin, ×165

Table 2. Relative frequencies of the different histopathological subgroups of tumours

Histopathological subgroup	Number of tumours	Percentage of all tumours
Adenomata	98	25.9
Carcinomata	280	74.1
Group 1	61	16.2
Group 2	152	40.2
Group 3	67	17.7

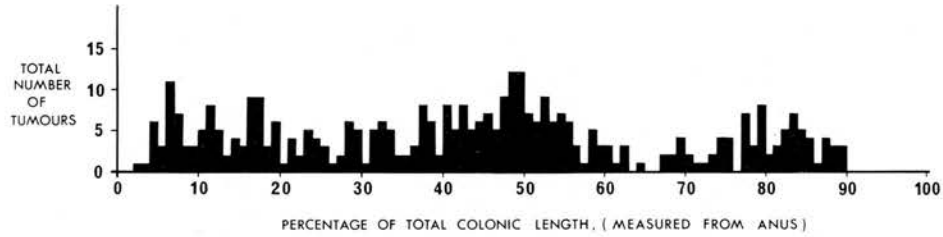

Fig. 9. Schematic representation of the distribution of all colonic tumours along the length of the bowel. Zero percent represents ano-rectal junction, and 100% represents colocaecal junction

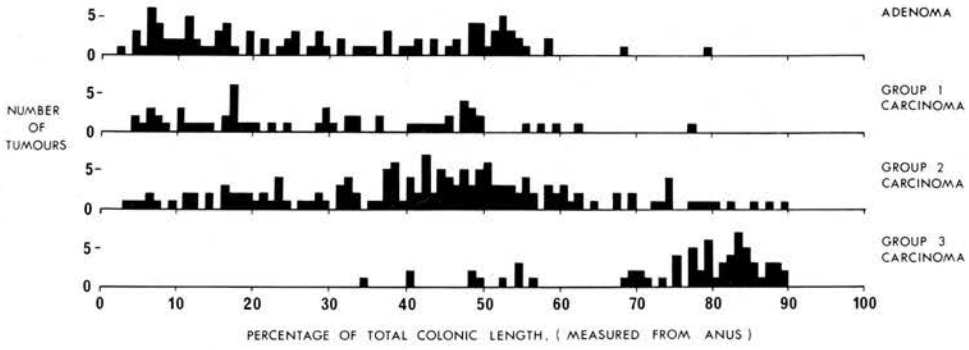

Fig. 10. Schematic representation of the distribution of the different types of tumour along the length of the bowel. Again 0% represents ano-rectal junction and 100% colocaecal junction

of these pooled figures shows that the proximal one tenth of the colon is tumour free, that the frequency is highest in the middle, and that there is something of a trough in the incidence of tumours at the junction of the proximal and middle thirds of the large bowel.

Examination of the distributions of individual subgroups of tumours is more revealing (Fig. 10). The benign tumours are largely confined to the distal three fifths of the large bowel, where their distribution is fairly uniform. The group 1 carcinomata, so similar histologically to the benign tumours, also have a similar distribution. Group 2 carcinomata on the other hand are seen through virtually the whole length of the colon, though with greatest frequency in the

Table 3. The mean number of the different kinds of tumour per rat at various times after the start of DMH injections

Duration of DMH treatment (weeks)	24	27 and 28	30	27–30
Number of rats	86	28	14	42
Number of tumours	217	97	43	140
Mean number of tumours per rat (total)	2.5	3.5	3.1	3.3
Mean number of adenomata per rat	0.7	1.1	0.3	0.8
Mean number of Group 1 carcinomata per rat	0.4	0.5	0.4	0.5
Mean number of Group 2 carcinomata per rat	0.9	1.6	1.8	1.7
Mean number of Group 3 carcinomata per rat	0.5	0.3	0.6	0.4

middle third. The poorly differentiated adenocarcinomata comprising group 3 have a highly distinctive distribution, being largely confined to the proximal third of the colon, a region where the incidence of other tumours types is low.

Numbers and Types of Tumours Related to Duration of DMH Treatment. In the preceeding sections we have considered as a whole all the colonic tumours occurring in the population of 142 rats killed at varying intervals up to 30 weeks after the commencement of DMH injections. The overall mean incidence of tumour is 2.7 per treated animal. Table 3 shows the mean number of tumours per rat at various times after the start of DMH injections. At 24 weeks the mean is 2.5, and the corresponding figure at 27 and 28 weeks is 3.5 tumours per animal. The difference between these two values is only just statistically significant; the trend is even less convincing when we consider that the value at 30 weeks is only 3.1 tumours per rat.

Table 3 also shows the frequency per rat of the different tumour types at the same time intervals. In general they are fairly similar save for an excess of Group 2 carcinomata at the later times.

Discussion

This study has confirmed that weekly subcutaneous injections of DMH at a dosage of 15 mg/kg body weight cause the development of colonic tumours in rats. This finding is in keeping with the experience of other authors (Druckrey, 1970; Wiebecke et al., 1973; Martin et al., 1973). The actual incidence of tumours by 24 weeks of treatment was just over 90%. The proximal tenth of the colon was invariably free of neoplasms, and the incidence was relatively low at the junction of the proximal and middle thirds of the colon, where the character of the mucosa changes (see below). This distribution is broadly similar to that noted by Pozharisski and coworkers (1975).

We have noted a histopathological spectrum of tumours similar to that described by many other authors, and have chosen to adopt a simple system of classification comprising only four basic tumour groups. Benign lesions showing no evidence of invasion of the muscularis mucosae were classified as adenomata. Lesions which differed only in that they showed localised invasion of the muscularis mucosae and submucosa were regarded as frankly malignant (Grinnell and Lane, 1958), and were categorised as group 1 carcinomata. Group 2 carcinomata were well differentiated or moderately well differentiated adenocarcinomata frequently showing deep invasion and surface ulceration. Group 3 carcinomata consisted of various histological types of poorly differentiated mucin-secreting adenocarcinomata. The distributions of the different tumour types are of particular interest. Adenomata (which comprised 26% of all tumours) are virtually confined to the distal half of the colon, a distribution which is shared by the group 1 carcinomata (16% of all tumours). Group 2 carcinomata (40% of all tumours) were distributed along most of the length of the bowel with a slight excess in the middle third, and group 3 carcinomata (representing 18% of all tumours) were conspicuously localised to the proximal one third of the colon. This curious distribution of different tumour types has been demonstrated by other authors (Ward, 1974; Reddy et al., 1976).

Ward (1974) sought to relate the differences in tumour frequency along the bowel to the obvious morphological differences. Recently we have demonstrated that the kinetic organisation of the colonic crypts in the rat colon varies from one site to another, using autoradiographic studies with tritiated thymidine (Sunter et al., 1978). It is possible that this variation affects the outcome of the carcinogenic stimulus.

Histopathological considerations suggest that the adenomata progress to group 1 carcinomata. It also seems likely that a proportion at least of group 2 carcinomata result from the further invasive growth of group 1 carcinomata. The close similarity of the anatomical distributions of these three categories of tumour supports these hypotheses. In contrast, the group 3 carcinomata seldom show histological evidence of evolution from tumours of a lower grade of malignancy; and, as we have established, the anatomical distributions are dissimilar. Thus there is a strong suggestion that group 3 carcinomata are poorly differentiated ab initio; or else the benign phase is transitory and rapidly superseded by the malignant growth.

The mean number of colonic tumours produced per rat seems to be of a similar order to that reported by several other authors (see for example Ward, 1974). Over a limited time period such slight increase as occurs in the mean number of tumours per affected rat can be attributed almost entirely to the increase in the number of group 2 carcinomata. This lends further support to our suggestion that these tumours are preceded by benign lesions, which are continually being formed. The malignant propensity of the DMH-induced adenomata parallels but probably exceeds that of isolated large bowel adenomata in man.

Acknowledgements. This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss. E. Wark who typed the manuscript and Mrs. E. Wallace and Mrs. M. Hughes who provided technical assistance. Mr. S. Brabazon prepared the illustrations.

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Received July 25, 1978

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KINETICS OF CHANGES IN THE CRYPTS OF THE JEJUNAL MUCOSA OF DIMETHYLHYDRAZINE-TREATED RATS

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Received 12 December 1977 Accepted 2 February 1978

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Summary.—When symmetrical 1,2 dimethylhydrazine was administered to rats by weekly s.c. injection, 37% of the animals had developed small intestinal carcinomas after 21–27 weeks. These lesions were largely localized to duodenum and upper jejunum. At the same time there was a diffuse crypt hyperplasia in the jejunum which affected all the treated animals, not just those with neoplasms. This marked hyperplasia was preceded by a modest sustained crypt elongation which was seen soon after DMH injections began.

In these hyperplastic jejunal crypts the absolute size of the proliferative compartment was increased, but the growth fraction calculated from labelling studies appeared to fall, probably by reduction in relative size of the proliferating population within the proliferative compartment.

No convincing alteration in actual cell-cycle time was observed in the abnormal crypts. There was a slight (25%) increase in cell-production rate in the abnormal crypts.

SYMMETRICAL 1,2-dimethylhydrazine (DMH) is a potent chemical carcinogen when administered to a variety of rodents. It is probably best known for its ability to induce colonic neoplasms, both in rats (Druckrey *et al.*, 1967) and in mice (Wiebecke *et al.*, 1969; Pegg and Hawks, 1971). Whilst injection of DMH appears to induce only colonic neoplasms in most strains of mice, it induces in rats a variety of other neoplasms as well. These occur less frequently than the colonic neoplasms, and include occasional hepatic and renal tumours (Druckrey, 1970) and tumours of the ear canal (Reddy *et al.*, 1975). Curious lesions of the stomach and biliary tree were described by Martin *et al.* (1973).

All these authors describe the additional occurrence of small-intestinal neoplasms in treated rats. The lesions are often malignant, and are usually located in the duodenum or the proximal part of the jejunum.

Apparently preneoplastic, or paraneoplastic, abnormalities have been described by several authors in the intestinal mucosa of DMH-treated animals. In the colon, ^{3}H-thymidine labelling is seen higher up the crypt in DMH-treated mice than in control mice (Deschner, 1974; Lipkin, 1974). In rats, there is a generalized increase in the height of the colonic crypts of treated animals (Wiebecke *et al.*, 1973) and in the circumference of the crypts (Tutton and Barkla, 1976). Such changes in the small-intestinal mucosa of DMH-treated animals have been less well recognized, although "mucosal hyperplasias" have been described (Wiebecke *et al.*, 1973).

In the present study DMH was administered to rats and changes in the crypts of the jejunal mucosa were monitored over the experimental period up to the development of frank neoplasia. Cytokinetic studies using tritiated thymi-

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dine (^{3}H-TdR) and vincristine have been carried out in an attempt to define some of the kinetic characteristics of the morphologically abnormal but non-neoplastic mucosae of treated animals. The data are compared with control data, and with normal values for animals from our colony.

MATERIALS AND METHODS

Animals and DMH-treatment regime.—Randomly bred female albino Wistar rats were used. At the beginning of DMH treatment the animals were 12–16 weeks of age and weighed 250–300 g. They were fed on standard rat cake (N.E. Farmers) and water *ad libitum*.

Symmetrical dimethylhydrazine dihydrochloride (Aldrich Chemical Co.) was administered by weekly s.c. injection at a dose of 15 mg (base)/kg body wt. The chemical was dissolved at a concentration of 1·66 g (of dihydrochloride) per 100 ml in normal saline containing 1·5% of EDTA added as a stabilizing agent. The solution was brought to a pH of 6·4 by the addition of N NaOH. It was freshly prepared each week. An interval of at least 1 week was observed between the final DMH injection and the killing of the animals, in order to avoid distortions arising from any acute effects of DMH on cell proliferation.

Crypt analysis during DMH treatment.—At various times after the start of DMH treatment (ranging between 4 and 27 weeks) small groups of 2 or 3 animals were killed by cervical dislocation. One hour before this the animals were given ^{3}H-TdR (Radiochemical Centre, Amersham), by i.p. injection at a dose of 0·5 μCi/g body wt; the specific activity of the ^{3}H-TdR was 5 Ci/m mol. A full necropsy was performed on each animal and particular attention was paid to the appearances of the small intestine and colon. These viscera were fixed for 6 h in Carnoy's fixative, and samples of small intestine were taken from a site just distal to the ligament of Treitz and from all small-intestinal tumours.

These transverse sections were processed through to paraffin wax and serial 3 μm sections were prepared and stained with haematoxylin and eosin and periodic acid/Schiff with and without amylase. Microautoradiographs were also prepared in the usual way (Al-Dewachi *et al.*, 1974). All neoplasms were categorized histologically into subgroups (see Results section). In the sections of jejunum, the "left" sides of 30 axially sectioned crypts were analysed per animal. The cell positions of labelled nuclei and of metaphase mitotic figures were recorded in terms of serial position counting upwards from Position 1 in the base of the crypt, together with the heights of individual crypts in cells (Cairnie, Lamerton and Steel, 1965); a cell was regarded as labelled if 5 or more grains were located over the nucleus (Wright, 1971). In each animal the data were projected on to a standard crypt the height of which was the mean crypt height of the animal concerned. The previously described modification (Wright, Morley and Appleton, 1972) of the method of Cairnie and Bentley (1967) to compensate for variation in crypt height was used to produce labelling and mitotic-index distribution diagrams. The circumference of crypts was measured by counting the number of cells appearing in transverse sections of crypts containing metaphases: "crypt column count". The mean of 50 such column counts was calculated, in each of 4 treated animals, and the inter-animal mean was determined.

All the animals in this experiment were killed at the same time of day (15.00 hours) in order to avoid any artefacts arising from diurnal variation (Sigdestad, Bauman and Lesher, 1969; Al-Dewachi *et al.*, 1976).

Frequency of labelled mitoses (FLM) studies.—Thirty rats which had received 24 weekly injections of DMH were given ^{3}H-TdR at a dose of 0·5 μCi/g body wt by i.p. injection at 0900 h. The animals were then killed serially at hourly intervals up to 12 h and then at 2-hourly intervals up to 50 h. Histological sections and autoradiographs were prepared as before from small-intestinal tumours and from macroscopically normal jejunum just distal to the ligament of Treitz. In the microautoradiographs of the jejunum, the crypt was divided for the purpose of analysis into cell-position groups, each group consisting of 4 cell positions. The lowest group consisted of Cell Positions 1–4, the second group Positions 5–8, and so on up the crypt. In each section a minimum of 20 mitotic figures was counted in each cell-position group, and the proportion of labelled mitoses was determined. In this way for each of the cell-position groups FLM curves were con-

structed, and the data were analysed by the method of Gilbert (1972).

Vinocristine studies.—Eighteen animals which had received 24 weekly injections of DMH were given vincristine sulphate (Oncovin, Eli Lilly) by i.p. injection at a dosage of 1 mg/kg body wt at 09.00 hours. The animals were then killed serially in groups of 3 at 20 min intervals up to 120 min after injection. Sections from upper jejunum and any tumours were taken and processed as before. Using serial histological sections from the jejunum, the "left sides" of 100 axially sectioned crypts were analysed in each animal. The cell position of arrested metaphases was recorded, as well as total crypt height. Adequacy of metaphase arrest was confirmed by the absence of any post-metaphase mitotic figures. As before, the data were projected on to a standard crypt, the height of which was the mean crypt height for all the animals in the vincristine group. This standard crypt was divided into cell position groups as described for the FLM experiment, and for each of these cell-position groups the cumulative mitotic index was plotted against time after vincristine.

From an analysis of 200 cross sections of jejunal crypts containing metaphases (50 from each of 4 animals) a correction factor was calculated to compensate for the overestimation of mitotic index due to migration of mitotic figures into the lumen of the crypt (Tannock, 1967).

RESULTS AND INTERPRETATION

Neoplasms

In animals killed after 21–27 weeks of DMH injections, the incidence of colonic neoplasms is virtually 100%. Most of the animals have several colonic tumours, and both benign and malignant types are represented.

By contrast the incidence of small-intestinal neoplasms is much lower: only 33 animals out of an initial series of 90 (*i.e.* 37%) developed small intestinal tumours. The longer the duration of DMH treatment the larger is the proportion of animals developing small-intestinal tumours. Again, in contrast to the large-bowel neoplasms, which are almost invariably multiple, the small-intestinal tumours are frequently solitary: only 4/33 affected animals manifested multiple tumours of the small bowel. Most of the small intestinal tumours (33/39) were situated within 40 mm of the pylorus, either in the most proximal part of the jejunum or in the distal duodenum. Animals with tumours in the distal jejunum or ileum had coexistent tumours in the upper small bowel. The reason for this curious localization of neoplasms in the upper small bowel appears to be that DMH is altered in the liver and secreted into the bile (Pozharisski *et al.*, 1975): thus the uppermost portion of the small bowel is exposed more intensely than the more distal portions to the carcinogenic stimulus.

Grossly, the small-bowel tumours are either ulcerated plaque-like lesions, or pearly, partly cystic, tumour nodules which grow through the muscular wall of the bowel. Histologically all the tumours are carcinomas. Four-fifths of the lesions have a tubulopapillary structure and, while invasion of the muscularis propria is a common feature, some of these lesions appear to be limited to mucosa and submucosa. Some of the tumours show formation of large central cysts, and most of the larger tumours have metastasized, at least as far as the regional lymph nodes. A smaller proportion of tumours (20%) are poorly differentiated mucin-secreting adenocarcinomas, showing a trabecular or acinar pattern, or a signet ring appearance.

Crypt hyperplasia

In occasional sections, examples are seen of gross atypia affecting one or two or a small group of crypt/villus units: we consider that these lesions represent established neoplasia. Quite different from these changes is the general and striking elongation of the intestinal crypts seen in animals after 24 weeks and more of DMH treatment (Fig. 1a and b). This remarkable crypt hyperplasia is seen both in animals bearing small-intestinal tumours, and in the much larger proportion of animals not so affected. In tumour-bearing animals,

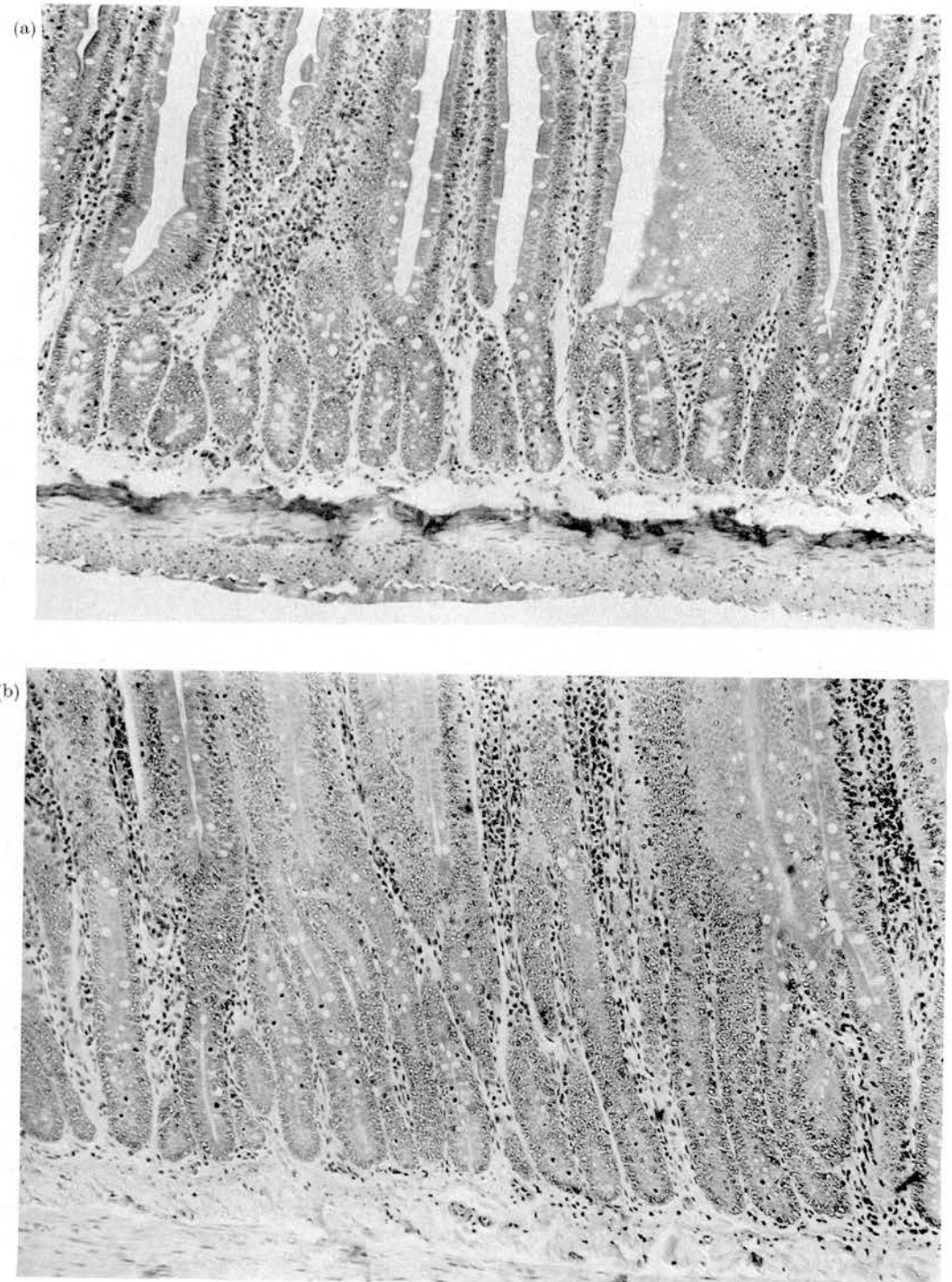

FIG. 1.—(a) Normal rat jejunal mucosa in a cross section of bowel. (b) The hyperplastic mucosa of an animal treated with DMH for 24 weeks. H. and E. ×125.

crypt hyperplasia is noted at sites remote from the tumours, and does not appear to be simply a reaction to the presence of a neoplasm.

Fig. 2 shows mean crypt height (in cells) plotted against the duration of DMH treatment in weeks. The mean crypt height of control animals is 33·7 cells, and this value is shown in the figure. Mean crypt heights of individual experimental animals are shown as dots, and the error bars represent ± s.e. mean. It can be seen that very soon after initiation of DMH treatment there is a modest sustained increase in the mean heights of crypts to 36–40 cells. Synchronously with the appearance of the first neoplasms at about 24 weeks of treatment, there is a further dramatic increase in mean crypt height, with individual means varying between 43 and 63 cells. This striking hyperplasia occurs in the absence of any features of atypia, and is generalized. Though the crypts may be virtually doubled in length, their circumference remains unaltered, the mean column count of treated animals (22·8 cells) not differing significantly from that of control animals (22·3 cells).

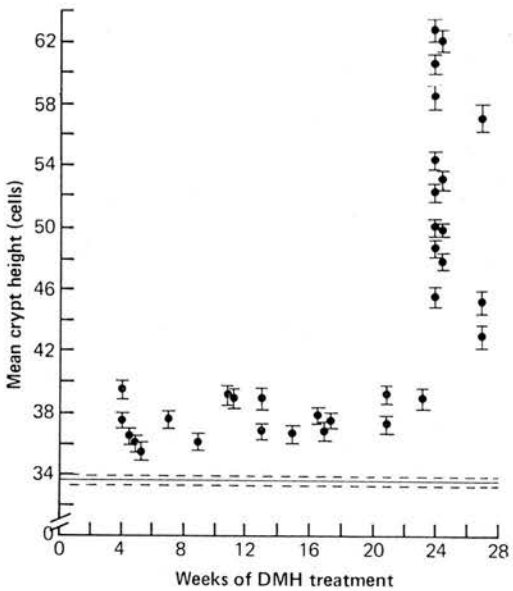

FIG. 2.—Mean crypt height (in cells) plotted against weeks of DMH treatment. Each dot represents an animal and the bars indicate s.e. The control value of mean crypt height is 33·7 cells, shown as a solid line flanked by broken lines indicating s.e.

^{3}H-TdR labelling

In control animals killed 1 h after injection of ^{3}H-TdR, the crude whole-crypt labelling index (I_S) is 26·3%. In Fig. 3 whole-crypt I_S is plotted against the duration of DMH treatment in weeks. Although values are probably slightly higher than the control level, there is no significant change apparent in these animals over the experimental period.

Fig. 4 shows the distribution of labelling activity within the small-intestinal crypts of one of the animals treated for 27 weeks with DMH. The mean I_S of each cell position has been plotted against cell-position number, and the curve has been fitted by eye. The shaded area indicates the 95% confidence limits of the labelling-index distribution curve of the control group of animals. In the treated animal the mean crypt height is 57 cells, as opposed to 34 in the control group. In general, the shape of the curve is similar, with low labelling indices in the basal cell positions, and higher values further up the crypt, declining to zero towards the top of the crypt. Several major differences are apparent, however: peak labelling index is much higher (around 70% as opposed to 48% in the control animals) and labelling

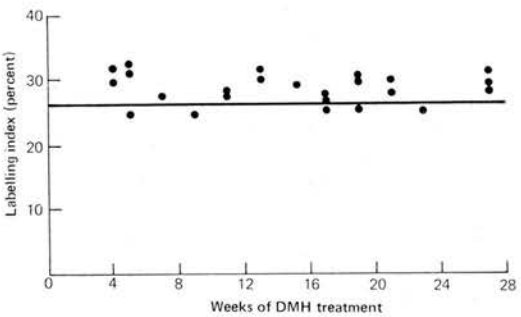

FIG. 3.—Mean labelling index (whole crypt) plotted against weeks of DMH treatment. Each dot represents one animal, and the control value of 26·3% is represented by the horizontal line.

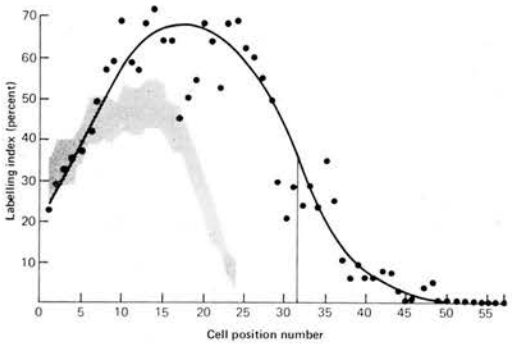

FIG. 4.—The labelling-index distribution curve of one of the animals treated for 27 weeks with DMH, with the confidence limits of the control group superimposed.

activity is seen much further up the crypt, in absolute though not in relative terms.

FLM experiment

Examples of the curves fitted by the Gilbert programme are shown in Fig. 5. Fig. 5a refers to Cell-position Group 1–4, and 5b refers to 9–12. Fig. 5c represents the pooled whole-crypt data. Table I summarizes the estimates of duration of the cell cycle (T_c) and the DNA-synthetic phase (t_s) in DMH-treated animals and in normal animals from the same colony (Al-Dewachi *et al.*, 1974).

The values of T_c in the proliferative compartment are virtually the same as in the normal animals. The pattern of relatively prolonged T_c in the basal cell-position groups is retained, although in Group 1–4 there may have been absolute shortening of the duration of T_c. It is difficult to be certain of this, since the standard errors generated by the Gilbert programme are almost certainly too small in this situation. For the most part, t_s appears to be only slightly prolonged by comparison with normal animals.

From labelling index data and the FLM curve, some estimate of the proliferating proportion, *i.e.* the growth fraction (I_p), can be obtained. Cleaver (1967) proposed that the cell position with 50% of the peak labelling index on the descending limb of the distribution curve of the labelling index, when compared with the total length of the crypt, gave some estimate of I_p. In the control group (see Fig. 4) a value of I_p calculated by this method is 0·59. This coincides very closely with an estimate of 0·61 obtained by Wright and his co-workers (1975). In the DMH-treated animals I_p calculated by this method is found to be only slightly lower than these normal values. In the curve illustrated in Fig. 4, for example, I_p is 0·56. Other animals killed at 27 weeks of treatment show I_p values of 0·56 and 0·55. Although there is an increase in the size of the proliferative compartment, there is

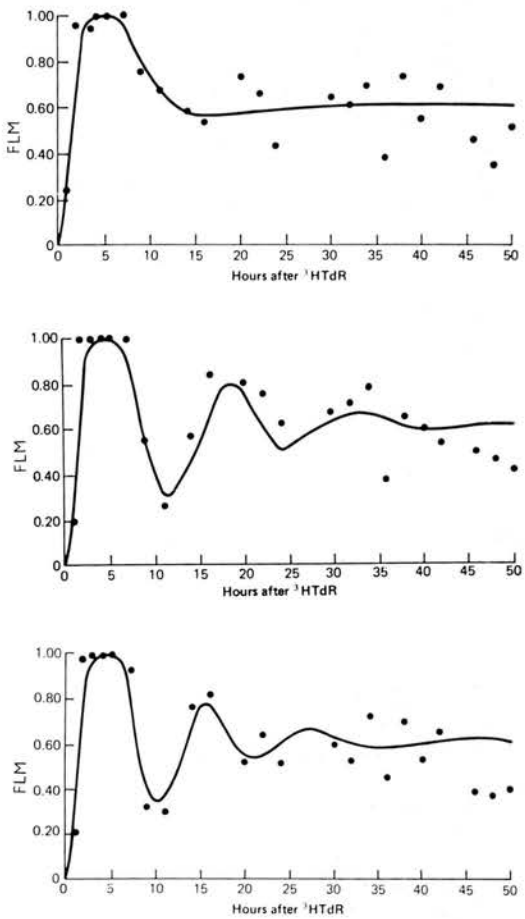

FIG. 5.—Examples of FLM curves: (a) Cell Position 1–4, (b) 9–12, (c) pooled whole crypt.

TABLE I.—*Summary of Estimates of T_c (cell-cycle time) and t_s (length of S phase) Derived from Computer Analysis of FLM Curves in Normal and DMH-treated Rats, Together with Standard Errors*

	DMH-treated		Control	
Cell position	T_c	t_s	T_c	t_s
1–4				
Mean ± s.e.	12·1 ± 1·4	7·4 ± 0·9	15·5 ± 0·2	8·6 ± 0·1
5–8				
Mean ± s.e.	13·4 ± 0·3	7·7 ± 0·2	12·3 ± 0·1	7·1 ± 0·1
9–12				
Mean ± s.e.	12·7 ± 0·2	7·9 ± 0·2	11·2 ± 0·1	6·4 ± 0·1
13–16				
Mean ± s.e.	10·6 ± 0·2	6·7 ± 0·2	10·8 ± 0·1	5·9 ± 0·1
17–20				
Mean ± s.e.	10·9 ± 0·1	6·6 ± 0·2	11·0 ± 0·2	5·9 ± 0·1
21–24				
Mean ± s.e.	10·3 ± 0·1	6·5 ± 0·2	10·7 ± 0·1	6·0 ± 0·1
25+				
Mean ± s.e.	10·7 ± 0·2	6·0 ± 0·3	—	—
Whole crypt				
Mean ± s.e.	10·9 ± 0·2	6·9 ± 0·1	11·3 ± 0·1	6·5 ± 0·1

therefore a suggestion that the growth fraction falls. A further method of calculating I_p is from the relationship:

$$I_p = I_{s\ obs}/I_{s\ exp}$$

where $I_{s\ obs}$ is the observed value for the labelling index, and $I_{s\ exp}$ a theoretical value derived from the cell-cycle parameters and the age distribution. The cell-cycle parameters have been calculated from the FLM curve. Using the data of Wright *et al.* (1975) and assuming an exponential age distribution, the estimate for I_p in normal animals is 0·67. In the DMH-treated animals this method gives estimates of I_p in the 27-week survivors of 0·48, 0·48 and 0·52. These figures are slightly lower than those derived from the labelling index distribution curves.

Vincristine study

Examples of the graphs of cumulative mitotic index against time are shown in Fig. 6: *a* refers to cell-position Group 1–4, *b* to Cell-position Group 9–12, while *c* is the pooled whole-crypt data. The lines have been fitted by least squares, and an exponential age distribution has been assumed; for comparison, the data of Wright (1974) have also been analysed on this assumption.

The correction factor to compensate for migration of metaphases towards the centre of the crypt (Tannock, 1967) was measured in 200 crypt cross-sections, and found to have a mean of 0·62. This is the same as that obtained by Wright (1974) in the normal rat.

In Table II the potential population-doubling times, also referred to as apparent cell-cycle times ($T_{c(a)}$), of DMH-treated animals are compared with the means calculated for normal rats. Tannock's factor has been taken into account, but no modifications have been made because of changes in the growth fraction. There is a common pattern of relatively long potential population-doubling times in the basal cells, with shorter $T_{c(a)}$ in the cell-position groups in the proliferative compartment. Higher up the crypt, $T_{c(a)}$ again becomes prolonged owing to the fall in growth fraction. Even with the imprecision of the stathmokinetic method, it is apparent that in all the cell-position groups within the proliferative compartment, $T_{c(a)}$ of treated animals is prolonged when compared with normal. Also, $T_{c(a)}$ of the whole crypt is prolonged in the treated animals when compared with normals.

This prolongation of $T_{c(a)}$ in DMH-treated animals is explicable on the basis of the falling I_p which was demonstrated by the labelling studies.

TABLE II.—*Summary of Apparent Cell-cycle Times ($T_{c(a)}$) in Normal and DMH-treated Rats, from Analysis of Metaphase Accumulation after Vincristine (Incorporating Tannock's Factor). The 95%-confidence Limits of $T_{c(a)}$ of the Whole-crypt Data are in Parentheses*

Cell position	DMH-treated animals	Control
1–4	41	15
5–8	18	9
9–12	14	8
13–16	12	8
17–20	11	11
21–24	11	23
25–28	14	71
29–32	14	—
33–36	14	—
37–40	23	—
Whole crypt	16·4 (11·8–27·2)	12·2 (10·2–15·0)

By constructing cumulative-birth-rate curves a measure of the cell velocity at the top of the crypt can be obtained (Cairnie *et al.*, 1965). A method has been described by Wright *et al.* (1972). In Fig. 7 cumulative-birth-rate curves of normal rats (data of Wright *et al.*, 1975) and of the DMH-treated animals are compared. These curves take into account the effects of Tannock's factor (q.v.). An exponential age distribution has been assumed. In the treated animals, cell velocity at the top of the crypt is 2·0 cell positions per hour, whereas in normals this value is 1·6/h. Thus we can conclude that the elongated abnormal crypts of the carcinogen-treated animals are producing a greater number of progeny than normal crypts. A comparison of the slope of the cumulative-birth-rate curves, however, shows that within the proliferative compartment the normal rises more steeply than the abnormal. Once again this effect appears to be a manifestation of a lower growth fraction within the proliferative compartment.

DISCUSSION

Crypt hyperplasia

These results show that during DMH treatment of rats crypt hyperplasia occurs in the proximal small bowel. There are apparently 2 separate phases: initially a sustained modest elongation is seen from control levels of about 34 cells to 36–40 cells; and after about 24 weeks of treatment a sudden, much more dramatic elongation to 43–63 cells is seen. This latter increase in crypt length coincides with the development, in an increasingly large proportion of animals, of foci of atypia and of frank malignant neoplasms largely localized to the region under study. This crypt hyperplasia is not explicable simply on the basis of ageing (Clarke, 1977), and it is tempting to speculate that it represents a preneoplastic phase.

Nevertheless, hyperplasia of small-intestinal crypts has been described in a variety of circumstances not obviously related to the development of neoplastic disease. Cairnie and Bentley (1967) described crypt hyperplasia as a normal physiological event during lactation. They found an increase in column count also, and a suggestion of increased migration rates; growth fraction remained unchanged.

As regards pathological states, gluten-sensitive enteropathy in man is characterized by crypt hyperplasia, associated with an increased rate of cell migration from the crypts (Wright *et al.*, 1973). It was considered on the basis of mitotic-index-distribution analysis that the growth fraction was slightly lower in coeliac patients than in controls, and there was good evidence of a shortening of the cell-cycle time in the proliferative compartment. Various workers have produced models of crypt hyperplasia in experimental animals. When rat jejunum is explanted to the skin surface a state of crypt hyperplastic villous atrophy results, with a greatly increased rate of migration of cells on to the surface of the mucosa (Loehry and Grace, 1974). Crypt hyperplasia has been described in rats infested with the nematode *Nippostrongylus brasiliensis* (Symons, 1965). Once again the migration rate of cells out of the crypts seemed to be enhanced. These conditions of crypt hyperplasia appear to represent a response to some irritant or inflammatory process

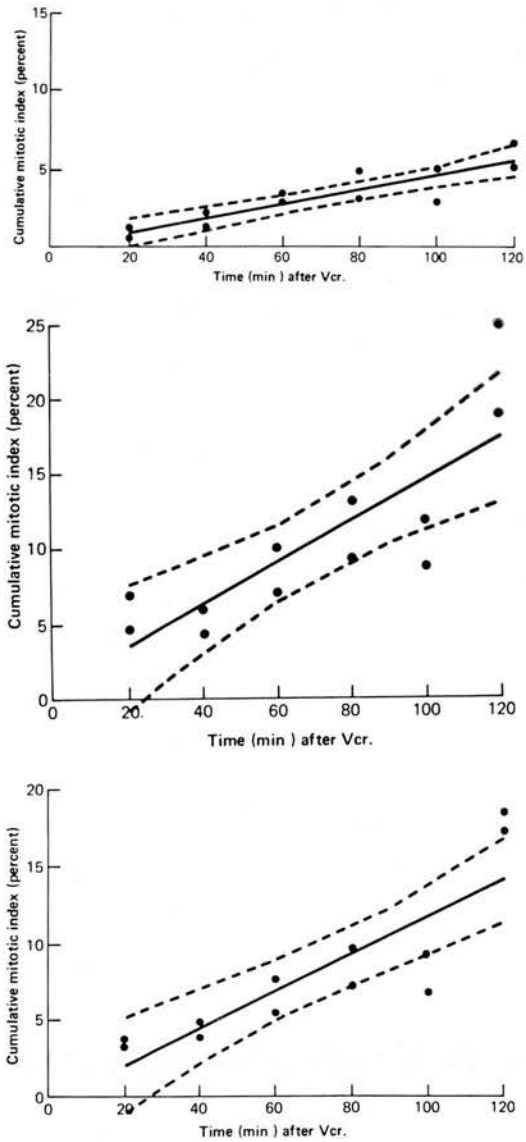

FIG. 6.—Examples of accumulation of mitosis after vincristine (Vcr.) administration: (a) Cell Position 1–4, (b) 9–12, (c) pooled whole crypt. The dotted lines indicate the 95% confidence limits for the fitted lines.

causing an increased rate of loss of mature cells from the mucosal surface.

Wiebecke *et al.* (1973) described "more or less localised mucosal hyperplasias" occurring in the small bowel of DMH-treated rats, and mentioned the presence of sharply demarcated zones of undifferentiated epithelium within the crypts. Mucosal hyperplasias have also been described in the colon of experimental animals during DMH carcinogenesis (Wiebecke *et al.*, 1973; Tutton and Barkla, 1976). In the latter authors' experience, increase in the circumference of the crypts was more prominent than elongation.

Growth fraction

From the form of the labelling-index distribution curves it is quite obvious that in the DMH-treated animals there is a considerable increase in the absolute size of the so-called proliferative compartment. However, the ratio of the site with 50% of the peak labelling to the total crypt height in fact falls from 0·59 in control animals to 0·56 in treated animals. This small change is probably not significant.

Considering the ratio of observed labelling index to theoretical labelling index (derived from FLM curves), we find an I_p estimate for the whole crypt of about 0·49 in DMH-treated animals, which is substantially less than the value of 0·67 calculated using data from normal animals from the same colony. This we consider reliable evidence that the growth fraction falls in the hyperplastic crypts of DMH-treated animals. Furthermore there is the strong suggestion that much of this reduction in proliferating population is occurring within the so-called proliferative compartment, and may be due to the alkylating properties of metabolites of DMH (Pozharisski *et al.*, 1975). There is, however, no histological evidence of cell

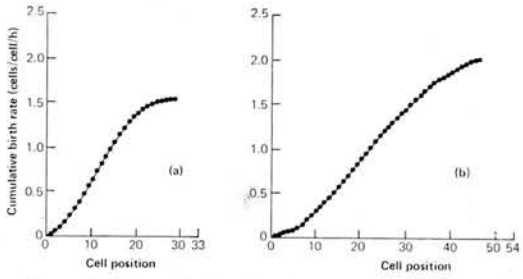

FIG. 7.—Cumulative birth-rate curves: (a) normal animals, (b) DMH-treated group.

damage or death. It is of interest to note that Loehry and Grace (1974) suspected a similar type of change, on the basis of simple labelling studies in their model. Wright and colleagues (1973) showed a fall in I_p in coeliac patients, while in lactating rats, Cairnie and Bentley (1967) thought I_p to be unchanged.

Cell proliferation

The results of the FLM experiment show that actual cell-cycle times remain more or less unchanged in treated animals. The small standard errors generated by the Gilbert programme are, we feel, a considerable underestimate in this situation. In the vincristine experiment a similar prolongation in $T_{c(a)}$ is seen to that noted by Tutton and Barkla (1976) in the colonic crypts of DMH-treated rats; $T_{c(a)}$ at virtually all sites is somewhat prolonged, and this is explicable on the basis of the fall in I_p. Cell-cycle times have seldom been estimated in other mucosal hyperplasias: in Nippostrongylus infestation, however, Symons (1965) found a fairly convincing reduction in T_c using an FLM method, and Wright *et al.* (1973) found suggestive evidence of a fall in $T_{c(a)}$ in human coeliac mucosa.

In all the hyperplasias described in the reports cited, an increase in cell production rate has been described or inferred. The results of the present work show a modest increase in the rate of movement of cells from the top of the crypt with a value of 2·0 cell positions/h in DMH-treated animals and 1·6 cell positions/h in controls.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss E. Wark who typed the manuscript, and Mrs E. Wallace and Mrs M. Hughes who provided technical assistance.

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KINETICS OF THE NON-NEOPLASTIC MUCOSA OF THE LARGE BOWEL OF DIMETHYLHYDRAZINE-TREATED RATS

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Received 5 January 1981 Accepted 26 March 1981

Summary.—Administration of 1,2 dimethylhydrazine (DMH) to rats by weekly s.c. injections causes the development of multiple epithelial tumours of the large bowel. These appear to arise as localized dysplastic abnormalities in hitherto apparently morphologically normal crypts. This study was undertaken in order to examine cell proliferation in such apparently normal crypts of DMH-treated animals.

A number of proliferative abnormalities are evident, including changes in the size of the crypts, changes in the disposition of proliferating cells within them and reduced cell-cycle times. The nature and the extent of the abnormalities vary from site to site along the length of the bowel, and reflect the vulnerability of the different segments of the bowel, not only to the carcinogenic effects of DMH, but also to short-term toxicity.

ADMINISTRATION of the carcinogen 1,2 dimethylhydrazine (DMH) to rats eventually results in the development of neoplasms in many organs (Druckrey, 1970). When it is given by repeated s.c. injections, a conspicuous organotropism is evident, with a high frequency of epithelial tumours of the intestinal tract (both small intestine and large bowel) and an acceptably low frequency of tumours elsewhere (Druckrey *et al.*, 1967; Druckrey, 1970; Martin *et al.*, 1973). Adjustment of the dosage schedule of the chemical can result in at least one colonic tumour in almost every treated animal by 20–30 weeks after the start of DMH treatment (Ward, 1974; Pozharisski, 1975; Maskens, 1976; Sunter *et al.*, 1978*a*). The microscopic structure of the tumours closely resembles that of human colonic neoplasms, and the model has been used quite extensively in the study of various aspects of colonic carcinogenesis, including the early stages of the morphogenesis of colonic cancer.

It is generally considered that frank neoplasia develops from areas of epithelial dysplasia or *in situ* neoplasia, such as have been described in many different situations in both experimental and human pathology. There is, however, some doubt as to the condition of the epithelial mucosa from which such dysplastic or pre-invasive lesions arise. For instance, Pozharisski (1975) stated that the development of malignant growths was not preceded by diffuse or focal hyperplasia of the mucosa, but he described abnormalities in the distribution of proliferating cells in otherwise normal crypts. On the other hand Wiebecke *et al.* (1973) described "more or less localized mucosal hyperplasias" in the colonic mucosa of DMH-treated rats. In the elongated crypts, the distribution of proliferating cells remained normal, but it was in such crypts that the earliest features of neoplasia were found. A progressive state of crypt hyperplasia, becoming more conspicuous with increasing duration of DMH treatment, has also been described (Maskens, 1976).

We have observed in the small-intestinal

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mucosa of DMH-treated rats a state of crypt hyperplasia which occurs separately from and apparently before the development of frankly neoplastic changes, and we have previously characterized this lesion in cytokinetic terms (Sunter *et al.*, 1978*b*). In the present study we have used the same kinetic techniques to examine the non-neoplastic colonic mucosa of DMH-treated rats, in order to explore the possibility of proliferative changes in such mucosa. The results have been compared with those for a historical control group of normal rats from the same colony (Sunter *et al.*, 1979) and with those for DMH-induced colonic adenocarcinoma (Sunter *et al.*, 1980).

MATERIALS AND METHODS

Animals and DMH-treatment schedule.—Randomly bred virgin female albino Wistar Porton rats from our own colony were used throughout. At the beginning of DMH treatment the animals were aged 12–16 weeks and weighed 250–300 g. They were fed on standard rat cake (N. E. Farmers, Aberdeen) and allowed tap water *ad libitum*.

A solution of symmetrical 1,2 dimethylhydrazine dihydrochloride (Aldrich Chemical Co.,) was administered by weekly s.c. injection, the dose being 15 mg (base)/kg body weight. The chemical was dissolved at a concentration of 1·66 g (of the dihydrochloride)/100 ml of normal saline, which contained 1·5% EDTA added as a stabilizer (Druckrey, 1970). The solution, freshly prepared each week, was brought to a pH of 6·4 by the addition of N NaOH.

At various times between 23 and 27 weeks after the start of DMH injections, animals were killed by cervical dislocation. In order to avoid distortions arising from the acute toxic effects of DMH on crypt cells, an interval of at last 1 week was allowed to elapse between the final injection of DMH and killing.

Crypt morphometry, labelling and mitotic indices.—Twenty-three weeks after the start of DMH injections 3 animals were killed, and a further 2 at 27 weeks. One hour before death, the animals had been given a single i.p. injection of tritiated thymidine [^{3}H]dT; Radiochemical Centre, Amersham, England) at a dose of 0·5 mCi/kg body weight; the specific activity of the [^{3}H]dT was 5 Ci/mmol. A full necropsy was performed on each animal, and the large bowel together with the caecum was removed, opened along its length and cleaned of faeces, then pinned mucosal surface uppermost to a cork board and placed in Carnoy's fixative for 6 h. The specimen was then transferred to 2 ethoxy-ethanol for a further 24 h before detailed inspection and dissection.

The total length of the colon was recorded, together with a description of the appearance and size of any tumours present, including a note as to their site. Transverse blocks of tumours were taken for histology and autoradiography, and the results have been reported elsewhere (Sunter *et al.*, 1978*a*; Sunter *et al.*, 1980). In addition to the blocks from the tumours, sections of tumour-free mucosa were obtained from 4 sites:

descending colon—30% of the distance from the anus to the ileocaecal valve;
transverse colon—60% of that distance;
ascending colon—90% of that distance;
caecum—the junction of the distal and middle thirds of the caecum.

These blocks were processed through to paraffin wax, and serial histological 3μm sections were prepared, and routinely stained with haematoxylin and eosin. Autoradiographs were prepared using a dipping technique; exposure was for 4 weeks, and following development the slides were stained with Harris's haematoxylin.

For each sample the "left sides" of 100 axial crypt sections were analysed, provided that gross inspection of the intact bowel and microscopic examination of the sections showed no evidence of a neoplasm capable of producing local distortions of crypt architecture. The total length of the crypt column in cells was recorded, together with the positions of mitotic figures and labelled epithelial-cell nuclei. The criterion for a labelled nucleus was 5 or more autoradiographic grains located directly over it. Using a modification (Wright *et al.*, 1972) of the method of Cairnie & Bentley (1967) diagrams showing the mean labelling and mitotic indices at each cell position in a crypt of mean length were produced for each sample.

Vincristine study.—After 24 weeks of carcinogen treatment 7 animals were given vincristine sulphate (Oncovin, Eli Lilly) by i.p.

injection at 09:00 at a dosage of 1 mg/kg body weight. Individual animals were then killed at 20 min intervals up to 140 min after the injection. Blocks were taken as before from tumour-free mucosa and serial sections were prepared and stained with haematoxylin and eosin. The "left sides" of 100 axial crypt sections were analysed, care being taken to avoid the immediate neighbourhood of tumours. The positions of arrested metaphases were recorded, together with the total length of the crypt column in cells. The adequacy of metaphase arrest was confirmed by the absence of post-metaphase figures in the material studied. The data were projected on to a standard crypt, the height of which was the mean for that particular site in the bowel. Thus a series of mitotic index distribution diagrams was obtained showing the accumulation of arrested metaphases at the various times after vincristine administration.

From the analysis of 200 cross-sections of crypts containing metaphases (50 cross-sections in each of 4 animals) the mean crypt-column count (the number of cell nuclei forming the circumference of the crypt) was calculated for each of the 4 selected sites in the bowel. From the same material, the correction factor to compensate for overestimation of mitotic index due to analysis of axially sectioned crypts was calculated (Tannock, 1967).

Fraction of labelled mitoses (FLM) study.—A further group of 31 animals at their 24th week of DMH treatment were given an i.p. injection of [^{3}H]dT at 0·5 mCi/kg body weight at 09:00, and single animals were killed at hourly intervals up to 14 h after the injection, and thereafter at 2 hourly intervals up to 48 h. Histological material was dealt with as described previously. For analysis of the autoradiographs the crypt was divided into cell-position groups each consisting of 4 consecutive cell positions; the lowest group consisted of the first 4 cell positions at the base of the crypt, the second group Positions 5 to 8, and so on up the crypt. In each sample at least 20 mitotic figures were located in each cell position group and the proportion of mitoses showing ^{3}H-labelling was determined. Thus an FLM curve for each cell position group was constructed. In the ascending colon it was impracticable to analyse crypts in this way because of the low mitotic index in the lowest cell positions, so the crypt was simply divided into an upper and lower half. At each site in the bowel an FLM curve for the whole crypt epithelium was constructed from the means of the values obtained in each of the component cell-position groups. The FLM curves were analysed by a modification of the computer method of Gilbert (1972).

RESULTS AND INTERPRETATION

A considerable number of samples were found on microscopic examination to include a part of a carcinomatous tumour or an adenomatous polyp. Because of local perturbations of crypt architecture that these lesions might have induced, such samples were excluded from morphometric and kinetic analysis. In addition, many other samples included crypts which showed obvious changes of epithelial dysplasia or even *in situ* neoplasia, such as have been described by other workers. These obviously abnormal crypts were also excluded from the analysis. The following observations therefore can be taken to apply only to colonic mucosa between tumours where the crypts have an essentially normal architecture and evidence of abnormality of cellular differentiation is minimal.

The morphometric data relevant to the crypts as a whole are summarized in Table I, along with the corresponding values we have observed previously in

TABLE I.—*Crypt morphometry and mitotic index (normal values in parentheses)*

Crypt parameters	Descending colon	Transverse colon	Ascending colon	Caecum
Mean lengths (cells)	46·5 (41·8)	50·4 (43·0)	29·6 (33·2)	31·1 (32·8)
Mean column count (cells)	22·2 (17·6)	22·0 (17·1)	22·6 (19·0)	24·6 (23·2)
Mean cell populations (cells)	1030 (735)	1110 (735)	670 (630)	765 (760)
Observed mitotic index	0·23 (0·56)	0·61 (0·48)	0·28 (0·59)	0·54 (0·55)
Tannock's factor	0·62 (0·63)	0·59 (0·63)	0·57 (0·63)	0·58 (0·56)
Corrected mitotic index	0·14 (0·35)	0·36 (0·30)	0·16 (0·37)	0·31 (0·31)

TABLE II.—*Kinetic data for the whole crypt calculated for the vincristine study and the mitotic index (normal values in parentheses)*

Parameter	Descending colon	Transverse colon	Ascending colon	Caecum
Birth rate (cells/1000/h)	14 (10)	10 (13)	10 (12)	17 (15)
Mitosis per crypt column	0·067 (0·15)	0·181 (0·13)	0·048 (0·12)	0·098 (0·10)
Cumulative k_B (cells/col/h)	0·67 (0·42)	0·51 (0·51)	0·31 (0·34)	0·50 (0·43)
Cell production rate (cells/h)	14·9 (7·3)	11·2 (8·7)	7·0 (6·5)	12·3 (9·9)
Mitotic duration (h)	0·10 (0·36)	0·35 (0·25)	0·15 (0·35)	0·20 (0·23)

normal animals from our colony (Sunter *et al.*, 1979). In the descending colon and transverse colon, treated animals show increases in both length and circumference of crypts, leading to a considerably increased cell population. In the ascending colon there are only minor changes in crypt length and circumference which do not change the overall size of the crypt cell population; in the caecum no significant changes are apparent. The means for the observed mitotic indices for the crypt as a whole show interesting deviations from the values obtained in normal animals. In the descending and ascending colon the indices are half normal, while those in the transverse colon and caecum show no obvious change. There are only minor changes in Tannock's factor, and the mitotic indices, corrected to take into account the geometric artefact introduced by the counting of axially sectioned crypts, confirm these results.

Table II shows the results from a consideration of the data derived from the stathmokinetic experiment and the corrected mitotic indices. For each site in the bowel the corrected whole-crypt mitotic index was plotted against time after vincristine. A rectangular age distribution was assumed for the crypt as a whole and a straight line was fitted by least squares. From the slopes of these lines, estimates of the cell birth rate were derived; they are similar to those we have obtained previously in normal rats, and this is perhaps surprising in view of the considerable reduction in mitotic index in treated animals in descending and ascending colon. The estimates of cumulative mitotic index in cells/column can be obtained from the product of mitotic index and mean crypt length; the fluctuations reflect changes in these variables.

From the distribution of arrested metaphases along the length of the crypt, at various times after the administration of vincristine, the accumulation of metaphases at each individual cell position has been monitored. We have assumed an exponential age distribution at individual cell positions; the estimates of cell birth rate have then been summed to yield a cumulative rate at the top of the crypt column (de Rodriguez *et al.*, 1979). This is the number of cells produced per column per hour, and is similar in treated and in normal animals, save that in descending colon it is somewhat increased in treated animals. The rate of cell production for the crypt as a whole can be evaluated as the product of cumulative cell birth rate and column count, and is shown in Table II; also shown are estimates of mitotic duration (de Rodriguez *et al.*, 1979). These show reductions in the duration of mitosis in descending and ascending colon, which would explain some of the apparent inconsistencies in the data.

The cell-cycle phase durations calculated from the curves fitted to the whole-crypt FLM data are shown in Table III. The durations of the S (t_S) and of the G_2 phase (t_{G2}) are not significantly altered from normal, but reduction in total cell-cycle time (T_c) has occurred at all 3 sites in the colon, owing to a reduction in the length of G_1 (t_{G1}). Despite these changes, the T_c values at the 3 colonic sites retain their normal gradient, with the shortest T_c in the ascending colon, an intermediate value in the transverse colon, and the longest in the descending colon. We have previously used the FLM technique to study

TABLE III.—*Kinetic data for the whole crypt derived principally from [^3H]dT labelling studies (normal values in parentheses)*

	Descending colon	Transverse colon	Ascending colon	Caecum
t_{G1} (h)	27·8 (46·6)	21·3 (30·7)	15·4 (24·6)	16·6 (15·2)
t_S (h)	8·9 (9·0)	8·3 (9·1)	10·3 (8·8)	7·9 (8·5)
t_{G2} (h)	2·4 (2·0)	2·2 (2·0)	1·4 (1·6)	1·8 (1·5)
T_c (h)	39·1 (57·9)	31·9 (42·0)	27·1 (39·3)	26·4 (25·4)
Coefficient of variation of T_c	45 (52)	37 (38)	29 (54)	31 (26)
Corrected theoretical labelling index	28 (21)	30 (24)	41 (32)	33 (26)
Observed labelling index	5·5 (7·1)	8·8 (7·2)	5·0 (7·3)	9·5 (10·9)
Growth fraction (%)	20 (34)	30 (30)	12 (23)	28 (30)
Total no. of proliferating cells/crypt	205 (250	325 (220)	80 (145)	220 (230)
Half-maximum position	20 (14)	29 (26)	19 (20)	13 (13)
Cut-off position	10 (11)	21 (17)	14 (16)	9 (10)

cell proliferation in the various histological types of colonic tumour in DMH-treated rats; the mean T_c values were all around 20 h (Sunter *et al.*, 1980)—even less than the estimates obtained in the present study on apparently healthy mucosa in DMH-treated rats.

Also shown in Table III are the estimates of the coefficient of variation of T_c provided by the Gilbert (1972) programme, and estimates of the theoretical labelling indices. In the calculation of this latter parameter we have assumed a rectangular age distribution, and have compensated for the variability of T_c (Appleton & Sunter, 1979). Mean labelling indices for the pulse-labelled animals are also shown, and the estimates of growth fraction (the proportion of cells proliferating) derived from the ratio of observed to theoretical labelling indices. These results show reductions in observed labelling index and growth fraction in descending and ascending colon. From the product of growth fraction and crypt-cell population the number of proliferating cells per crypt can be calculated. In descending and ascending colon there is a reduction in the number of proliferating cells per crypt (which in the descending colon has occurred despite a moderate increase in the actual size of the crypt), while the enlarged crypts in the transverse colon show a proportionate increase in the number of proliferating cells.

An indication of the distribution of proliferating cells along the length of the crypt is obtained from the labelling-index distribution diagrams which have been constructed for each sample taken from the group of pulse-labelled animals. The figure shows the pooled data for all 5 animals for each of the 4 sites in the bowel. The shaded areas indicate 95% confidence limits for the points obtained by pooling data from a group of 4 control animals. All the individual samples from a particular site showed broadly similar features. In the descending colon, where the labelling index as a whole is somewhat reduced in treated animals, it appears that the distribution of labelled cells is altered, with a reduction in labelling index throughout the proliferation zone and extension of labelled cells into higher cell positions within the slightly lengthened crypt. The pattern of a lower labelling index in the basal cell positions is lost. In the transverse colon where labelling index as a whole is somewhat enhanced, it is in the highest (and to some extent the lowest) cell positions that the increased numbers of labelled cells are found. In the ascending colon there is a fairly symmetrical small reduction in labelling index, and in the caecum no change is seen, as might be anticipated in view of the absence of any other significant changes. An indication of the size of the compartment within the crypt where there are proliferating cells

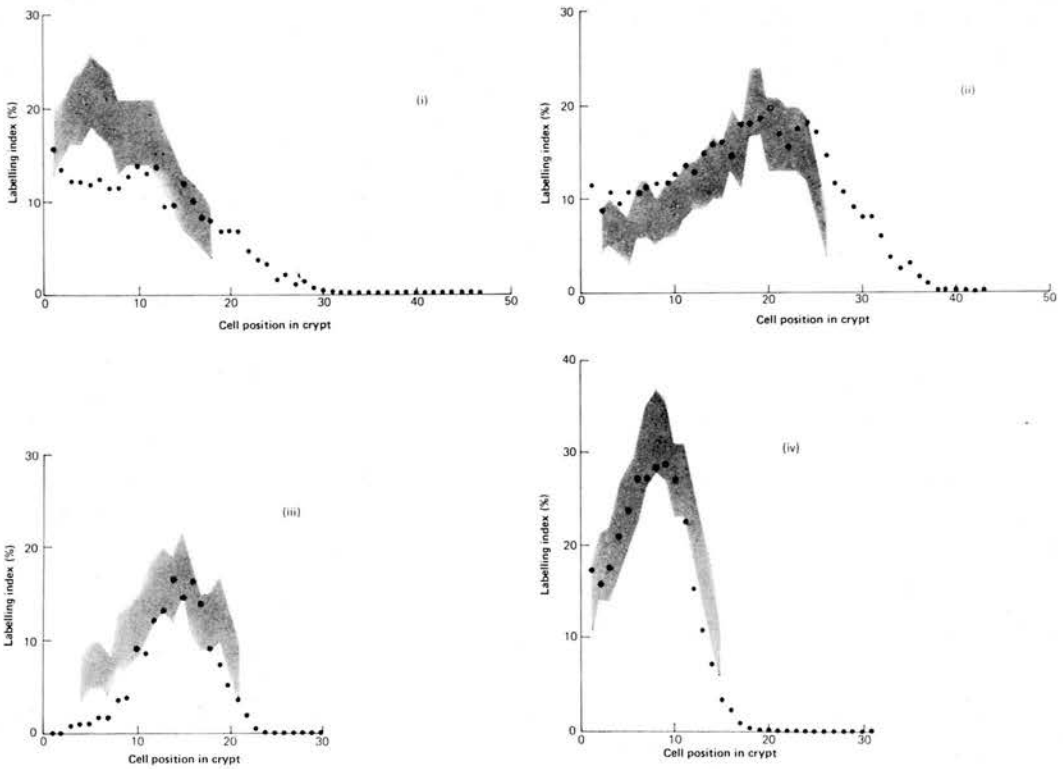

FIGURE.—Labelling-index distribution for (i) descending colon, (ii) transverse colon, (iii) ascending colon and (iv) caecum, obtained by pooling the data from 5 pulse-labelled animals. The shaded areas indicate 95% confidence limits for the points obtained by pooling data from a group of 4 control animals.

may be gained by the position at which labelling index is reduced to half its maximum value (Cleaver, 1967) or by the cut-off position (Cairnie *et al.*, 1965; Appleton *et al.*, 1981) and both these parameters are estimated in Table III.

The estimates of the cell-cycle times (T_c) calculated for the separate four-cell position groups are shown in Table IV, along with the corresponding estimates we have obtained previously in normal animals. T_c is consistently reduced in every cell position group at all 3 sites in the colons of the treated animals. This consistency is reassuring in view of the unreliability of the standard errors estimated by the Gilbert programme. In the caecum there is no convincing evidence for a change. In those sites where changes are evident the usual distribution of T_c within the crypt is still retained, the longest times occurring at the base of the crypt.

We have been dealing with differences in parameter estimates between treated and control rats in terms of their size, and not of their statistical significance. It is indeed difficult to assess the standard errors of some of the quantities estimated, such as the cut-off position, or the cell-cycle-phase durations, and thus the theoretical labelling index and growth fraction; but for the more straightforward variables we can perform t tests between the means of treated and control groups. For example the increase in crypt length in descending colon is 4·7 cells $\pm$ 0·97 (s.e., calculated from 14 control animals and 11 treated)—a significant increase.

TABLE IV.—*Cell-cycle times (h) at different sites along the length of the bowel, calculated by cell-position groups within the crypt (normal values in parentheses)*

Cell-position group	Descending colon	Transverse colon	Ascending colon	Caecum
1–4 (base)	41·4 (68·4)	53·0 (70·7)		30·2 (34·6)
5–8	40·0 (61·0)	37·5 (64·4)	28·2 (39·7)	25·1 (25·4)
9–12	35·5 (64·9)	45·2 (39·8)		23·8 (23·4)
13–16	38·4 (45·9)	32·8 (38·5)		23·4 (24·1)
17–20	36·6 (53·5)	29·9 (38·7)	26·9 (32·1)	
21–24	33·5 (71·5)	27·0 (37·4)		
25+		26·4 (—)		

The estimate of the cumulative cell-birth rate has a standard error of 0·03 in descending colon of both control and treated rats, if we ignore any possible error in the estimation of Tannock's factor and are satisfied as to the adequacy of metaphase accumulation and of the assumption of an exponential age distribution at each cell position. Trying to take the non-systematic elements of these other sources of error into account, *e.g.* by postulating a relative standard error of 10% in Tannock's factor, increases the standard error of the difference, but this in fact still leaves the difference (0·67–0·42 = 0·25) significantly different from zero, the new estimate of its standard error being 0·8. This indicates that the size of the experiment is such that differences which look worthy of discussion probably also attain statistical significance.

DISCUSSION

In the normal rat the morphology and cytokinetic organization of the mucosal crypts vary quite considerably from one site to another in the large bowel and the caecum (Sunter *et al.*, 1979). Additionally, the various segments of the bowel show different degrees of vulnerability to the long-term carcinogenic effects of chemicals of the cycasin group (Pozharisski, 1975; Maskens, 1976; Nomura *et al.*, 1978; Sunter *et al.*, 1978*a*). These two considerations make the assessment of the possible preneoplastic nature of the changes in the mucosal crypts rather complex. Further problems are caused by the appearance of lesions variously described as dysplasias, adenomatous changes in glands, and *in situ* neoplasias, which are generally considered to be the immediate precursors of frank neoplasms. We have tried to exclude such lesions in this study, which has been designed to document any generalized changes in apparently normal areas of mucosa in DMH-treated rats.

Changes in size crypt

Pozharisski (1975) stated that in his experience the abnormal mucosal crypts in the large bowel of DMH-treated rats were of a normal length, and could not therefore be properly regarded as hyperplastic. Wiebecke *et al.* (1973) and Ward (1974) described focal hyperplastic lesions in the colon, from which early neoplasms appeared to originate. On the other hand Maskens (1976), using material from the descending colon, showed a steady and progressive increase in the length of the mucosal crypts, which appeared diffuse. Again, using material from the descending colon, Tutton & Barkla (1976) found that, while crypt length did not alter significantly during DMH treatment, there was a considerable increase in crypt circumference, leading to an increase in cell population from 1300 to 1800 cells. In the present study we have found increases in crypt length and crypt-column count in transverse colon and descending colon, with an increase in population size similar to that observed by Tutton & Barkla (1976). The minor changes we have noted in the ascending colon effect no significant change in crypt-population size, and in caecum there is little evidence of change

in any of these parameters. In the consideration of overall crypt size, it is of note that Nomura *et al.* (1978) described areas of mucosal atrophy in addition to areas of hyperplasia.

Distribution of proliferating cells within the crypt

While a change in crypt length was not a feature in the material of Pozharisski (1975), his studies showed a considerable lengthening of the zone in which ^{3}H-labelled cells were seen. This increase in the absolute size of the proliferation zone has been commented on by others (Wiebecke *et al.*, 1973; Maskens, 1976; Tutton & Barkla, 1976). Increased labelling indices were apparent in the study of Wiebecke *et al.* (1973) but not in that of Maskens (1976). In our material too there appears to be an increase in the absolute size of the proliferation zone, as evidenced by the changes in half-maximum position in the descending and transverse colon. Whole-crypt labelling indices are reduced in the ascending and descending colon but perhaps increased in the transverse colon. The net results of the changes are essentially a loss of the normal pattern of a lower index in basal cell positions than higher up the crypt in transverse and descending colon, and a slight symmetrical reduction in the ascending colon. The caecum does not show any significant change.

Rates of cell proliferation

In the descending and ascending colon the mitotic index is much lower in treated animals than in controls. This, coupled with the data derived from the stathmokinetic experiment with vincristine, used to calculate a cumulative birth rate, suggests a surprising fall in the duration of mitosis in these 2 sites. This rather unexpected finding will require further investigation in subsequent studies.

Crypt cell production rate, a measure of the net output of cells per crypt, is clearly increased in the descending colon, but the significance of the changes in transverse colon and caecum is much less certain.

The FLM experiment shows a clear and consistent pattern of a reduced cell-cycle time (T_c) in treated mucosa at all 3 colonic sites. The reduction is effected through G_1, other cell-cycle phases showing no great changes. Analysis of the data by cell-position groups shows that cells at all levels in the crypt are affected similarly, but that the usual distribution T_c within the crypt is retained. Overall, the values for T_c in the DMH-treated mucosa are intermediate between those of the normal mucosa and those of DMH-induced colonic tumours (Sunter *et al.*, 1980). The estimates of growth fraction in the whole crypt show reductions in ascending colon, with no change in the transverse colon. Given that the overall size of the proliferation compartment is increased in descending and transverse colon and unchanged in the ascending colon, the figures suggest that the reduction in the growth fraction of whole crypt is largely a result of a fall within the proliferation zone itself, a situation we have previously observed in the small bowel mucosa of DMH-treated rats (Sunter *et al.*, 1978*b*).

CONCLUDING REMARKS

This study has shown that there are a number of morphometric and cytokinetic abnormalities in the morphologically normal-looking crypts in the large-bowel mucosa of rats exposed to chronic DMH. The most obvious abnormality is an increase in the numbers of cells in the crypts, which is confined to the transverse colon and the descending colon; in the ascending colon, although there are morphometric abnormalities, no significant alteration in crypt-cell population is seen, and in the caecum there is no change in morphometric indices. Changes in the distribution of proliferating cells are apparent in transverse and descending colon. The normal patern of a low labelling index in the basal cell-position groups becomes less obvious and the proliferation

zone extends further up the crypt, both in absolute and relative terms. Again the ascending colon is less severely affected, only a slight general reduction being apparent. No significant abnormality is seen in the caecum. The severity of these changes seems to reflect very closely the vulnerability of the intestinal mucosa at different sites to the chronic carcinogenic effects of DMH; in our material caecal tumours are virtually never seen, tumours in the proximal part of the ascending colon are rare and tumours in the rest of the bowel are very common (Sunter *et al.*, 1978*a*). Similar selective vulnerability to the acute toxic effects of DMH is seen (Sunter *et al.*, 1981).

At all 3 colonic sites there is a substantial and consistent reduction in cell-cycle time, brought about by a reduction in the duration of G_1. The growth fraction is reduced in descending and ascending colon, while in the transverse colon the estimate is the same as for normal animals. However, given the changes in the size of the proliferation zone, it is apparent that even at this site there must be a substantial reduction in growth fraction within the proliferation zone, just as in descending and ascending colon. The caecum shows no obvious changes of proliferative indices calculated from thymidine-labelling studies.

It is a matter for speculation whether the kinetic abnormalities described represent a specific preneoplastic state or a nonspecific response to a toxin; work is in progress to try to define these mucosal changes using other techniques.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Miss E. Wark and Miss Y. Allen who typed successive versions of the manuscript, Mrs E Wallace who provided technical assistance, and Mr R. Joyce who prepared the illustration.

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CELL PROLIFERATION OF COLONIC NEOPLASMS IN DIMETHYLHYDRAZINE-TREATED RATS

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Received 8 February 1980 Accepted 20 March 1980

Summary.—We have measured mitotic indices and ^{3}H-thymidine-labelling indices for the colonic epithelial tumours induced in rats by the administration of dimethylhydrazine (DMH). The fraction-of-labelled-mitoses (FLM) technique has been used to estimate the duration of the cell-cycle phases.

In general, mitotic and labelling indices in the tumours are similar to those in the proliferation zone of the normal crypt epithelium; lesions considered to be least well differentiated on histological grounds appear to have the lowest mean labelling index. Benign tumours and the different types of malignant tumours have mean cell-cycle times about half those of the normal mucosa.

THE PARENTERAL ADMINISTRATION of symmetrical 1,2-dimethylhydrazine (DMH) to several species of rodents causes the development of a number of different types of neoplasm. Tumours of the intestinal tract are particularly frequent, and this striking organotropism, first commented upon by Druckrey *et al.* (1967) in their studies on rats, has been amply confirmed by many other workers, both in rats (Wiebecke *et al.*, 1973; Martin *et al.*, 1973; Ward, 1974; Pozharisski, 1975) in mice (Wiebecke *et al.*, 1969; Pegg & Hawks, 1971; Haase *et al.*, 1973; Toth *et al.*, 1976) and in hamsters (Winneker *et al.*, 1977). The induced intestinal tumours are almost always of epithelial origin. In rats, tumours occur both in the small intestine and in the colon, whereas in mice the colonic neoplasms predominate overwhelmingly. It is as a highly selective colonic carcinogen that DMH has found most of its experimental applications.

Several reports have described in some detail the range of histopathological types of colonic tumours arising in DMH-treated rats (Wiebecke *et al.*, 1973; Ward, 1974; Pozharisski, 1975; Maskens, 1976). We have recently described a simple morphological classification of these different tumour types (Sunter *et al.*, 1978*a*) which are strikingly similar to the colonic neoplasms in humans. In the rat model, however, purely villous adenomas are never seen, and carcinomatous neoplasms outnumber adenomatous neoplasms by about 3 to 1. An interesting finding is that each tumour type has a characteristic distribution along the length of the rat colon, with the least-differentiated carcinomas concentrated proximally, whilst adenomas occur mainly in the distal half. To a certain extent these differences in distribution mirror the variation in morphology and kinetic organization of the rat colonic mucosa from one site to another (Sunter *et al.*, 1978*b*).

In the present study we have examined cell proliferation in the various types of colonic tumour arising in DMH-treated rats. Techniques used have included calculation of the mitotic index (I_m) and the ^{3}H-thymidine ([^{3}H]-TdR)-labelling index (I_s), together with estimation of the major cell-cycle parameters from fraction-

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of-labelled-mitoses (FLM) experiments. The different tumour types have been compared in terms of these parameters, both with one another and with the normal colonic mucosa in the region where each type was most prevalent (Sunter *et al.*, 1979).

MATERIALS AND METHODS

Animals and treatment schedule.—Randomly bred virgin female Wistar Porton rats from our own colony were used throughout. They were fed on standard rat cake (N. E. Farmers, Aberdeen) and allowed tap water *ad libitum*. Injections of DMH were begun when the animals were aged 12–16 weeks and weighed 250–300 g.

A solution of symmetrical 1,2-dimethylhydrazine dihydrochloride (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was administered at weekly intervals by s.c. injection at a dose of 15 mg (of the base)/kg body wt. The chemical was dissolved at a concentration of 1·66 g (of dihydrochloride)/100 ml of normal saline with 1·5% EDTA added as a stabilizing agent, and brought to a pH of 6·4 by the addition of N NaOH solution. The solution was freshly prepared each week.

Labelling and mitotic indices.—At various times between 23 and 30 weeks after the start of DMH injections, when most animals would be expected to have developed one or more colonic neoplasms, small groups of up to 6 rats were given an i.p. injection of [^{3}H]-TdR (Radiochemical Centre, Amersham) at a dose of 0·5 mCi/kg body wt. The specific activity of the [^{3}H]-TdR was 5 Ci/mmol. All the injections were given at 14:00 to minimize possible artefacts due to diurnal variation (Chang, 1971; Hamilton, 1979). One hour after the injection the animals were killed by cervical dislocation. The large bowel was removed, opened along its length and cleaned, pinned mucosal surface up to a cork board, and fixed for 6 h in Carnoy's solution. The specimen was then transferred to Cellosolve for a further 24 h before detailed inspection and dissection. At least 1 week was allowed from the final injection of DMH until killing, to permit recovery from the acute toxic effects of the chemical (Deschner, 1978).

The total lengths of the colons were recorded, together with a description of the naked-eye appearances of any tumours, including their longitudinal and transverse diameters, measured with Vernier calipers, and their sites in terms of percentage distance from the anus. A complete transverse block was taken from each tumour and this material was processed through to paraffin wax. Histological sections 3 μm thick were prepared. Sections were routinely stained with haematoxylin and eosin and in some cases by the periodic-acid–Schiff method after treatment with amylase. These sections were used for histopathological diagnosis and classification. Autoradiographs were prepared from other sections using a dipping technique; the exposure period was 4 weeks and, after development, the slides were stained with Harris's haematoxylin.

For each of the different tumour types, specific zones within the tumour, showing particular morphological features, were analysed (for details see Results section and Fig. 1). In each zone sampled, 3000 neoplastic epithelial cells were counted, and the percentages of mitotic (late prophases, metaphases and anaphases) and of [^{3}H]-TdR-labelled cells (5 or more grains over the nucleus) were calculated. The mean values for the different zones in the different types of tumours were compared with each other, and with those for the normal mucosa (Sunter *et al.*, 1979) by means of t tests.

Fraction of labelled mitoses studies.—After 24 weeks of DMH treatment 3 successive groups of animals, each of about 25 individuals, were given a single i.p. injection of [^{3}H]-TdR at a dose of 0·5 mCi/kg body wt. In the first group animals were killed singly at hourly intervals up to 14 h after injection and thereafter at 2 h intervals up to 48 h. Histological sections and autoradiographs were prepared as described in the previous section. After the tumours had been classified it was found, not surprisingly, that adequate examples of all the tumour types did not appear at all the sampling times on the FLM experiment; the subsequent groups of animals were used in an attempt progressively to fill in these gaps. These efforts met with varying degrees of success, but inevitably the curves remain incomplete for the less frequent tumour types.

Because of the practical difficulties in locating a sufficiently large number of mitotic figures in the small strictly defined zones, it was necessary to count whole cross-sections of tumour; the Group 2 carcinomas were considered in 2 portions, "superficial" and

"deeply infiltrative". For each area 100 mitotic figures were analysed and the proportion showing ^3H labelling was determined. FLM curves were constructed, and the data were analysed using a modification of the method of Gilbert (1972).

RESULTS

Colonic tumours and their distribution

We have recently published our observations on the histopathological features, classification and distribution of the colonic tumours occurring in several experimental groups of DMH-treated rats (Sunter *et al.*, 1978*a*) and our further experience of the model has confirmed the consistency of the described pattern. The results will therefore be only briefly summarized here.

By 24 weeks of DMH treatment, over 90% of rats had developed one or more colonic neoplasms. The lesions were frequently multiple: at 24 weeks there was a mean of 2·5 tumours per rat, and this figure increased to 3·3 by between 27 and 30 weeks of treatment. The tumours were distributed over the full length of the colon, save for the proximal 10 mm or so. They fell readily into a simple system of classification:

Adenomas with a tubular or tubulovillous microscopic pattern constituted about a quarter of the total. Their distribution was virtually confined to the distal half of the colon.

Group 1 *carcinomas* formed ~16% of all tumours, and were virtually indistinguishable microscopically from adenomas, save for the presence of infiltrating neoplastic tubules extending through the muscularis mucosae. These lesions shared the distribution of the adenomas.

Group 2 *carcinomas* formed 40% of the tumours, were distributed throughout the length of the colon (most in the middle third) and consisted of moderately differentiated adenocarcinoma, often showing at the periphery what appeared to be residual areas of "adenomatous" tissue.

Group 3 *carcinomas* formed ~18% of the tumours, and consisted of a group of poorly differentiated adenocarcinomas showing, in contrast to the other tumour classes, a variety of histological appearances: a small-celled acinar or trabecular pattern, signet-ring-cell carcinoma and colloid carcinoma. Often several histological patterns were seen in a single tumour. These lesions were conspicuously localized to the proximal one-third of the colon and, unlike the other carcinomas, tended to metastasize to regional lymph nodes and throughout the peritoneum.

Labelling and mitotic indices

Fig. 1 shows a schematic representation of a typical adenoma. Two zones within these lesions were selected for the assessment of I_m and I_s. "Zone a" consisted of a 0·05mm-thick strip of tissue at the luminal surface, whilst "Zone b" was a 0·1mm-thick strip of tissue 0·05mm internal to "Zone a". The distances were measured with an eyepiece graticule. A number of lesions found at several different times after the start of DMH injections, and ranging in size between 3 and 6 mm in greatest diameter, were analysed and the results are presented in Table I. There is some variation in I_m and I_s of each zone between individual tumours, but a striking and consistent difference is seen between surface and subsurface zone. The mean I_s at the surface is 4% and in the subsurface region 24%. That this difference is not due simply to a failure of [^3H]-TdR to reach the surface is suggested by parallel differences in I_m.

Mean values for I_m and I_s at several sites within the several types of carcinoma are compared in Table II. The figures represent the means of 6–7 individual tumours found at different times after starting DMH treatment. In no case was there any correlation of a proliferative index with duration of DMH treatment. In the Group 1 carcinomas the mean mitotic and labelling indices in the subsurface region are similar to the values obtained for the groups of invasive tubules situated within and deep to the

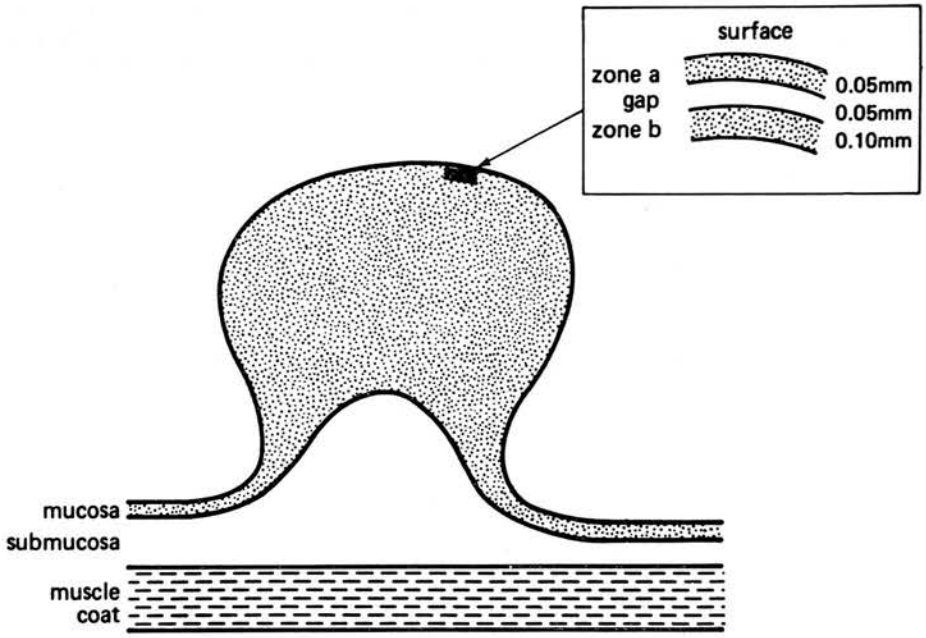

FIG. 1.—Schematic representation of a benign pedunculated adenoma.

TABLE I.—*Mitotic and labelling indices (as %) in 2 zones in a number of benign adenomas examined at various times after DMH treatment began*

Weeks after start of DMH	Surface "Zone a"		Subsurface "Zone b"	
	I_m	I_s	I_m	I_s
23	0·3	2·0	0·9	23·3
24	0·3	3·4	1·5	22·3
24	0·3	3·1	0·8	20·1
27	0·2	7·5	1·1	22·1
27	0·2	4·4	0·7	28·8
28	0·9	6·4	1·4	24·5
Mean	0·36	4·47	1·06	23·6
s.e.	0·11	0·86	0·20	1·4

muscularis mucosae, on whose presence the histological diagnosis rests. In both zones values are similar to those seen in the subsurface zone of adenomas. The small differences between the mean values of I_m and I_s in the several zones within Group 2 carcinomas are not statistically significant. However, the mean I_s of the Group 3 carcinomas is significantly lower than that of either the subsurface zone of the adenomas or the deep central zone of Group 2 carcinomas.

Within many of the zones in the different tumour types minor local fluctuations in the labelling index were seen; labelled cells often appeared in clusters of 3–4 cells. This might imply either local fluctuations in the rate of cell proliferation, or a degree of local synchrony.

The labelling indices of the tumours and of the position with maximum labelling within the proliferation compartment of normal colonic crypts at the site of maximal tumour frequency are similar. An exception to this rule is the deep central region of Group 2 carcinomas, wherein I_s is significantly higher than the maximum I_s of the antecedent crypts.

FLM studies

Fig. 2 shows the FLM data for the different tumour types, and the theoretical curve corresponding to a cell-cycle time (T_c) of 20 h, the durations of G_1 (t_{G_1}), DNA-synthesis (t_S) and G_2 (t_{G_2}) being 10·5 h, 7·5 h and 2 h respectively. The duration of mitosis is equally shared between G_1 and G_2. The coefficients of

TABLE II.—*Mean mitotic and labelling indices (as %) at various sites within the different tumour types, compared with the labelling indices in the normal mucosal crypts at sites in the colon corresponding to the sites of maximal incidence of each tumour type.*

		Neoplasms		Normal colon I_s	
Type of tumour	Site within tumour	I_m	I_s	Whole crypt	Maximum
Adenoma	Surface (Zone a)	0·36	4·5		
	Subsurface (Zone b)	1·03	23·6		
Group 1 carcinoma	Subsurface	1·22	20·3	7·6	21
	Invasive glands deep to m. mucosae	1·11	21·7		
Group 2 carcinoma	Peripheral shoulder	0·84	21·3		
	Deep central region	1·09	26·0	6·9	18
	Invasive glands in m. propria	0·82	19·6		
Group 3 carcinoma	Trabeculae and acini of small cells	0·62	15·6	5·5	17

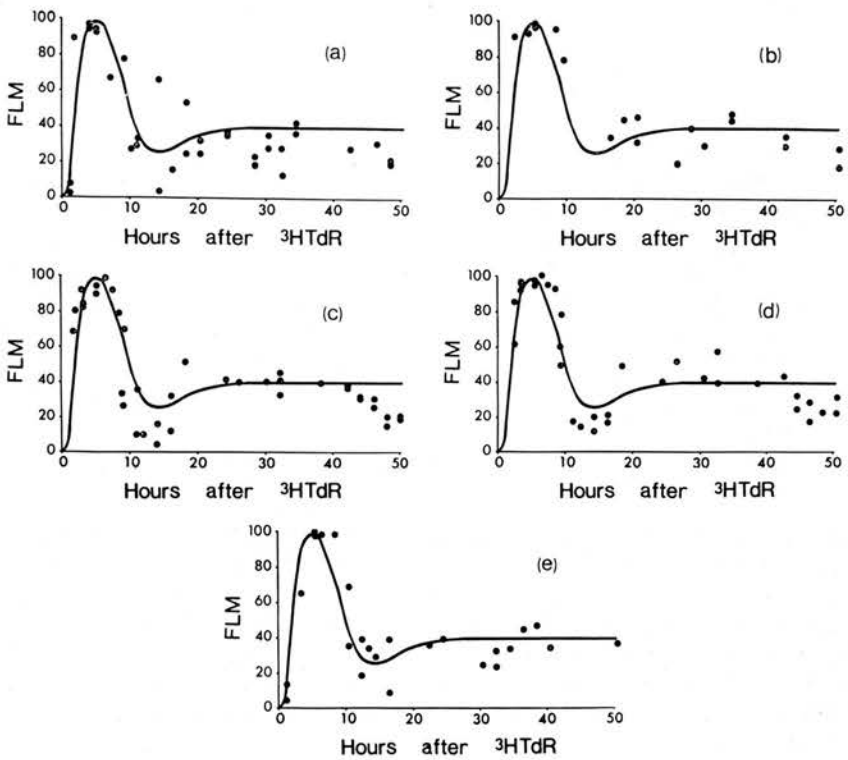

FIG. 2.—FLM data for the different tumour types; (a) adenomas, (b) Group 1 carcinomas, (c) Central region of Group 2 carcinomas, (d) deeply invasive region of Group 2 carcinomas, and (e) Group 3 carcinomas. The curve shown in each case is for the case $T_c = 20$ h, $t_{G_2} = 2$ h, $t_S + G_2 = 9{\cdot}5$ h. Coefficients of variation for T_c, t_{G_2} and $t_S + G_2$ are 50%, 50% and 25% respectively.

variation of T_c, t_{G_2} and t_{S+G_2} are 50%, 50% and 25%. The incompleteness of the basic data provides considerable problems in obtaining good estimates of the cell-cycle parameters. We have modified the method of Gilbert (1972) to deal with such sparse data by changing the way in which the numerical integration is done, but there is inevitably a tendency for fitted curves to be linear between widely spaced points, and if points are absent from the first descending limb and trough, this can greatly affect the fitted parameters, as can be seen by inserting a single arbitrary reading where none exists. We believe it is of more interest that the mean cell-cycle times in the different tumour types are similar, and about half that of the normal mucosa, than that they may show small variations between themselves. However, the T_cs found from "best fits" to the 5 sets of data in Fig. 2 were respectively 21, 18, 17, 18 and 26 h; the values for normal colonic crypts were 35, 42 and 58 h in the ascending, transverse and descending colon respectively (Sunter *et al.*, 1979).

DISCUSSION

The study of spontaneous or induced primary tumours in experimental animals and in man is often hampered by the unobtrusiveness of the lesions and their inaccessibility. In the present study it was usually impossible to tell whether a given animal was suffering from a colonic tumour before killing, because the colonic lesions were almost always asymptomatic. Any obvious illness was usually due to a small-intestinal tumour, or a tumour at some non-colonic site. Experience showed, however, that by 24 weeks of DMH treatment over 90% of animals harboured one or more colonic tumours, so we did not consider it necessary to resort to laparotomy or radiographic examination (Rosengren, 1978) before committing groups of treated animals to kinetic experiments. However, the fact that there were several different histopathological types of tumour necessitated the use of relatively large numbers of animals killed in several groups to obtain an adequate number of points on the FLM curves. Despite the large number of animals used there are still some gaps. Furthermore, because of the unpredictable nature of the individual response to the carcinogen there is considerable variation in the size of the neoplasms, giving rise to difficulties in interpretation.

Zonal variations in proliferative indices within tumours are well recognized (Hermens & Barendsen, 1967; Aherne *et al.*, 1977) and one of the factors involved in this is the proximity of the vascular supply (Tannock, 1968). In the present study I_m and I_s in the subsurface regions of the adenomas were several times greater than the values found at the surface of the lesion. Remoteness from the blood supply, or exposure to toxic substances in the bowel lumen, may account for this difference, but histological evidence of necrosis or inflammation was lacking. Neither was there evidence of surface differentiation, which would also account for loss of proliferative activity. In the various types of carcinoma, I_s was similar to the maximum I_s in the mucosal crypts of normal bowel corresponding to the site of maximum tumour incidence, and fluctuations of values between tumours was not great. It is difficult to compare our results with those of other workers, because the cells counted and the analytical methods may differ in important details, but Schauer *et al.* (1971) reported a mean I_s of 37·5% in the "proliferative zone" of DMH-induced adenocarcinomas in rats compared with 23·9% in the "normal crypts base", whilst Wiebecke *et al.* (1973) observed maximum labelling indices of about 30% in adenomatous polyps which was "nearly twice as high as in normal mucosa". These latter workers also described a zone of increased labelling at the invasive base of malignant polyps, a feature which we have not observed. In the zones we have examined, I_s is certainly about 3 times the I_s for the whole crypt in normal rats, but is of the same order as in the most proliferative part of the crypt.

Several previous studies have provided information on the duration of the phases of the cell cycle in these experimental tumours. Schauer *et al.* (1971) found, as we did, that t_s was virtually the same in the colonic tumours as in normal colon; their estimate of T_c in the tumours was 21·4 h, but their value of 32·3 h in the surrounding colonic mucosa is less than in our normal animals. Using a colchicine–TdR technique, Pozharisski & Klimashevski (1974) showed that there was considerable variation in the cell-cycle parameters from one tumour to another; their estimate of 28·1 h for the mean t_s in adenocarcinomas is extremely high, and their estimate of 53·6 h for the "generation time" is not comparable with our "cell-cycle time", because of lack of information about the growth fraction. Tutton & Barkla (1976), using a stathmokinetic technique, showed that cell birth rates in colonic tumours were slightly lower than those in the positions of fastest cell proliferation in the normal crypt, but were comparable to the levels seen in the crypt base.

Our FLM study provides convincing evidence that the mean cell-cycle time in the induced tumours is about half that in the normal colonic mucosa crypts (Sunter *et al.*, 1979) and, given the values of I_s, this implies a considerable decrease in the growth fraction in the tumours compared to the proliferation zone of the crypts. We have also observed a reduction in growth fraction in the state of hyperplasia which precedes neoplasia in the small bowel (Sunter *et al.*, 1978*c*); in this preneoplastic state no change in T_c was evident. It is clear from these observations that documentation of the precise sequence of cytokinetic changes during the neoplastic transformation of the crypt epithelium will lead to a better understanding of the early stages of tumorigenesis.

This work was supported by a grant from the North of England Council of the Cancer Research Campaign. We would like to thank Mrs E. Wallace for technical assistance, Mr S. Brabazon who prepared the illustrations and Miss E. Wark who typed the manuscript.

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Virchows Arch [Cell Pathol] (1981) 36:47–57

Virchows Archiv B
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Acute Changes Occurring in the Intestinal Mucosae of Rats Given a Single Injection of 1,2 Dimethylhydrazine

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Summary. In the long term, administration of dimethylhydrazine (DMH) to rats results in the development of tumours in both small intestine and colon. This study has been undertaken in order to document the sequence of changes occurring in the intestinal mucosa in the first 108 h following a single subcutaneous injection of DMH.

After a lag of several hours there is evidence of damage to cells in the proliferation zone of the intestinal crypts, and a brief reduction in tritiated thymidine labelling index. A phase of compensatory regenerative activity emerges from the setting of continuing cell damage, resulting in restoration of the mucosa to normal.

The severity of the toxic damage to the intestinal mucosa at various sites mirrors the vulnerability of the mucosa to the long term carcinogenic effects of DMH, suggesting that inherent properties of the mucosa may be of more importance than other cocarcinogenic influences in the ultimate development of tumours.

Key words: DMH – Intestine – Toxicity – Mucosa

Introduction

The parenteral administration of symmetrical dimethylhydrazine (DMH) to rats results in the development of benign and malignant epithelial tumours of small and large bowel (Druckrey et al. 1967; Ward 1974). The small intestinal tumours occur predominantly in duodenum and upper jejunum (Druckrey 1970; Martin et al. 1973; Ward 1974), but there is some variation in distribution of tumours in the large bowel. For example, some groups have reported a substantial incidence of caecal tumours (Ward 1974; Pozharisski 1975; Maskens 1976; Lindström et al. 1978) while others have rarely noted them (Nomura et al.

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0340-6075/81/0036/0047/$02.20

1978; Sunter et al., 1978b; Castleden and Shilkin 1979). On the other hand, it is generally agreed that tumours arise infrequently in the proximal ascending colon. As it is now clear that the active metabolites of DMH are distributed principally by the bloodstream, localisation of tumours to particular parts of the gut must be the result either of tissue-specific vulnerability to the particular effects of these metabolites, or of the cocarcinogenic effects of other agents acting at a local level. Such cocarcinogens could include bile acids, faecal sterols, bacteria and other components of the bowel contents.

The present study has been undertaken in order to document the sequence of changes which occur in the intestinal mucosae of the rat following a single subcutaneous injection of DMH. These changes include the acute toxic effects of the chemical and the subsequent phase of compensatory responses which restore the mucosa to an apparently normal state within four or five days of the injection. The techniques employed have included morphology, simple morphometry and tritiated thymidine (^{3}HTdR) labelling with preparation of autoradiographs. Samples of mucosa have been analysed both from areas which we have previously found to be extremely vulnerable to the long-term carcinogenic effects of DMH, and from areas found to be resistant to these effects; the vulnerability of these two types of mucosa to the acute toxic damage have been compared. The degree of damage should give a clearer indication of innate tissue susceptibility to the effects of the carcinogen than longterm studies on the incidences of tumours, since such studies may be influenced conspicuously by the presence of low-grade chronic cocarcinogenic factors.

Materials and Methods

Eighty-four randomly-bred virgin female albino Wistar Porton rats were used. The animals were aged between 12 and 16 weeks when the experiments were carried out and weighed between 225 and 300 g. They were fed on standard rat cake (N.E. Farmers) and were allowed tap water ad libitum.

The animals were divided into a low-dose DMH group and a high-dose DMH group. The former were given a single subcutaneous injection of symmetrical dimethylhydrazine dihydrochloride (Aldrich Chemical Co.) at a dosage of 15 mg (base)/kg body weight (the same dose that we have used in our experiments on the chronic effects of DMH), and the latter a single injection of 60 mg (base)/kg body weight. The solutions were prepared by dissolving the chemical in normal saline at a concentration of 1.66 g/100 ml for the low dose and 6.64 g/100 ml for the high dose. A 1.5% concentration of EDTA was included as a stabilising agent (Druckrey 1970) and the solutions were brought to a pH 6.4 by the addition of N sodium hydroxide solution.

Groups of three animals were killed after various time intervals up to 108 h following the injection of the carcinogen, each animal having been given an intraperitoneal injection of tritiated thymidine (Radiochemical Centre, Amersham, England) 1 h previously. The specific activity of the ^{3}HTdR was 5 Ci/mMole and the dose used was 0.5 mCi/kg body weight. Groups of untreated ^{3}HTdR-labelled animals were killed at 0900 h, 1700 h and 0100 h at the beginning of the experiment to serve as controls; the several time points were deemed necessary to obviate the possibility of confusion resulting from diurnal variation in crypt size and proliferative indices (Al-Dewachi et al. 1976; Hamilton 1980).

The animals were killed by cervical dislocation and the whole of the intestinal tract removed. The large bowel, including the caecum, was opened along its length, cleaned and pinned to a cork board mucosal surface uppermost. Both the large bowel and the entire unopened small bowel were then fixed for six hours in Carnoy's fluid. They were then transferred to 2-ethoxyethanol where they remained for a further 24 h prior to the selection of blocks, which consisted of transverse

sections including the full thickness of the bowel wall. The *upper jejunum*, just distal to the ligament of Treitz, and the *distal colon* at a site 30% of the distance from the anus to the ileocolic junction were selected as sites which we have found to be very vulnerable to the long-term carcinogenic effects of DMH. The distal portion of the *caecum*, and the *proximal colon* 90% of the distance from the anus to the ileocolic junction, were selected as two sites which in our material have seldom developed tumours after DMH administration. This material was processed through to paraffin wax. Serial transverse histological sections were cut at 3 μm thickness; some of these were stained with haematoxylin and eosin, or by the periodic acid-Schiff method after amylase treatment, while others were for the preparation of microautoradiographs using a dipping method, the exposure period being four weeks.

In each autoradiograph a quantitative analysis was carried out using the 'left sides' of thirty perfect axial sections of crypts. The total length of each crypt in cell units, the crypt column, was recorded together with the cell position of labelled nuclei, the criterion for the identification of labelling being five or more grains located over the nucleus. In each group of three animals the data were combined to produce mean values for the crypt column count and for a whole crypt labelling index; using the method of Cairnie and Bentley (1967) as modified by Wright et al. (1972), labelling index distribution diagrams were produced.

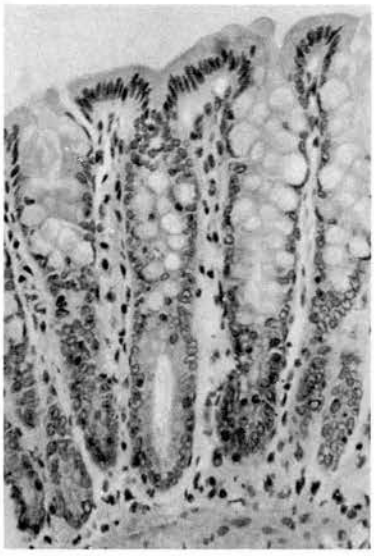
Results

Animals were killed in groups of three after various time intervals up to 56 h after DMH in the case of the low-dose group (30 animals), and up to 108 h in the case of the high-dose group (45 animals). Evidence of local discomfort at the injection site was minimal, and the low-dose animals showed no evidence of systemic illness following treatment. The high-dose group became anorectic in the two days following DMH and lost a little weight. Then appetite and weight were rapidly restored and no animals were lost as a result of DMH administration. The macroscopic findings at postmortem examination of the low-dose animals were entirely normal, and the only abnormality noted in the high-dose group was the unusual emptiness of the colon in the two days following injection, and the mild hyperaemia of the mucosa.

Morphological Changes

The sequence of microscopic changes in the intestinal mucosae was similar in type and distribution in both the low-dose and high-dose groups. In the former the changes were subtle and of a minor degree, whereas in the latter they were much more conspicuous. Only the morphological changes in the high-dose group will therefore be described and illustrated in detail.

In the animals examined 2 h and 4 h after the administration of DMH no morphological abnormality was noted in any of the samples of bowel. This delay is presumably accounted for, at least in part, by the time taken for the DMH to be absorbed from the injection site, be distributed and converted into active intermediary metabolites. At 8 h evidence of crypt cell damage was seen in the form of an accumulation of pyknotic and karyorrhectic nuclei confined to that region of the crypt where actively proliferating cells are normally found (Fig. 1). This damage was conspicuous in both the distal colon and the upper jejunum, sites very vulnerable to the long-term carcinogenic effects of DMH, but was also seen in the caecum and the proximal colon where tumours arise much less frequently. The nature of the damage was such that its accurate quantification was not considered feasible, but, in the initial stages



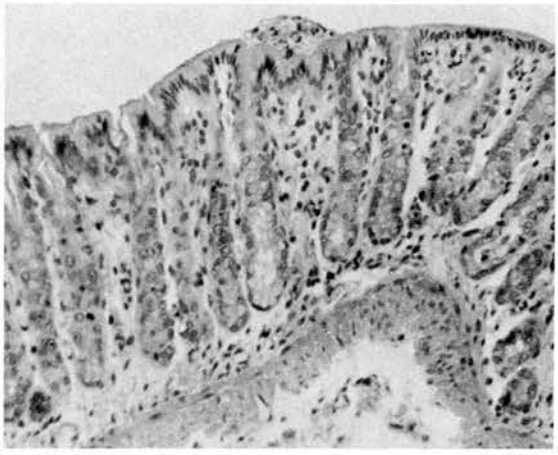


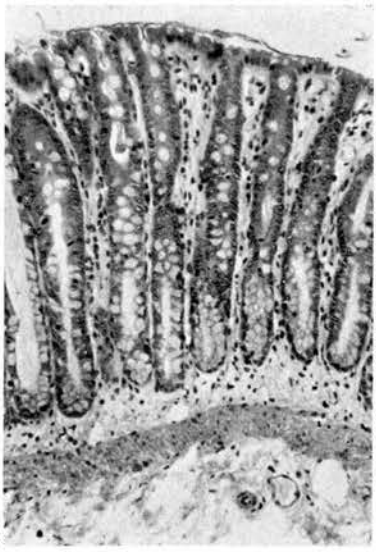
Fig. 1. Distal colon 8 h after high-dose DMH showing the accumulation of pyknotic and karyorrhectic nuclei in the lower part (proliferation zone) of the crypt. H&E × 180

Fig. 2. Distal colon 56 h after high-dose DMH showing depletion of cells in the crypt column, and abnormalities of the surviving cells. H&E × 120

at least, it seemed to be of a similar order in the two types of mucosa. Evidence of continuing cell death seen in successive groups of animals led to a progressive depopulation of crypt cells in the distal colon and the upper jejunum but not apparently in the other two sites (vide infra). The depleted crypts were seen to be shortened and lined by a reduced number of cells, giving a curious moth-eaten look to the crypt lining (Fig. 2). At the same time as the development of these obvious changes in crypt morphology there occurred an influx of inflammatory cells into the lamina propria, invariably of a minor degree and apparently short-lived.

From this setting of continuing crypt cell death and mucosal damage there emerged a phase of regenerative activity. The cells lining the crypt became enlarged and somewhat irregular in shape with darkly staining cytoplasm and large nuclei (Fig. 3). Mitotic figures, having virtually disappeared from the tissue in the period immediately following DMH injection, were seen once again in large numbers and were not confined to the site of the normal proliferation zone of the crypt. Occasional abnormal mitoses were seen. This compensatory proliferative activity led to a transient state of crypt hyperplasia in the upper jejunum (vide infra) but no such phenomenon was consistently observed in the distal colon, possibly because the observation period was too short. By the end of the experimental period crypt microarchitecture was restored essentially to normal however, even in these areas of bowel most affected by the acute toxic effects of the chemical.

The changes in the architecture of the small-intestinal villi have not been subjected to detailed morphometric analysis but a clear pattern of response



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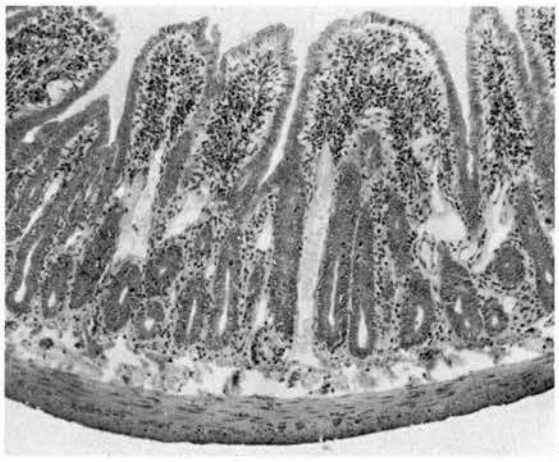

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Fig. 3. Distal colon 96 h after high-dose DMH showing regenerative changes with restoration of a normal crypt architecture largely completed. H&E × 120

Fig. 4. Proximal jejunum 72 h after high-dose DMH showing a picture of crypt hyperplasia and partial villous atrophy. H&E × 80

has emerged. In the initial 24 h following DMH injection, when evidence of crypt cell damage was clear, the villus cells remained normal and villus architecture was also apparently normal. In the period succeeding this there was a progressive shortening of the villi; presumably, since villus cell damage was not observed, this was due to an inadequate efflux of cells from the crypts resulting in a state of partial villous atrophy which was at its most severe 72 h after DMH (Fig. 4). Restoration of villus architecture ensued and was essentially completed by 108 h.

Morphometry and Thymidine Labelling

Figure 5 demonstrates the fluctuations in mean crypt length (in cells) occurring in the distal colon and the upper jejunum at various times after DMH. Both low-dose data and high-dose data have been illustrated, along with an indication of the overall mean ± 1 standard deviation of the mean values derived from all nine control animals, there being no consistent pattern of diurnal variation in this material. Even at the low-dose level there was significant depopulation of the crypts which was compensated for in the upper jejunum by 56 h but not in the distal colon. In the high-dose group depopulation was more severe in both sites but was largely compensated for by 108 h, an overshoot in the response having occurred in the upper jejunum but not in the distal colon. In contrast the caecum and the proximal colon showed no significant fluctuations in mean crypt column length at either dose level (Fig. 6), suggesting either an extremely effective compensatory response or that little damage had been sustained in the first place.

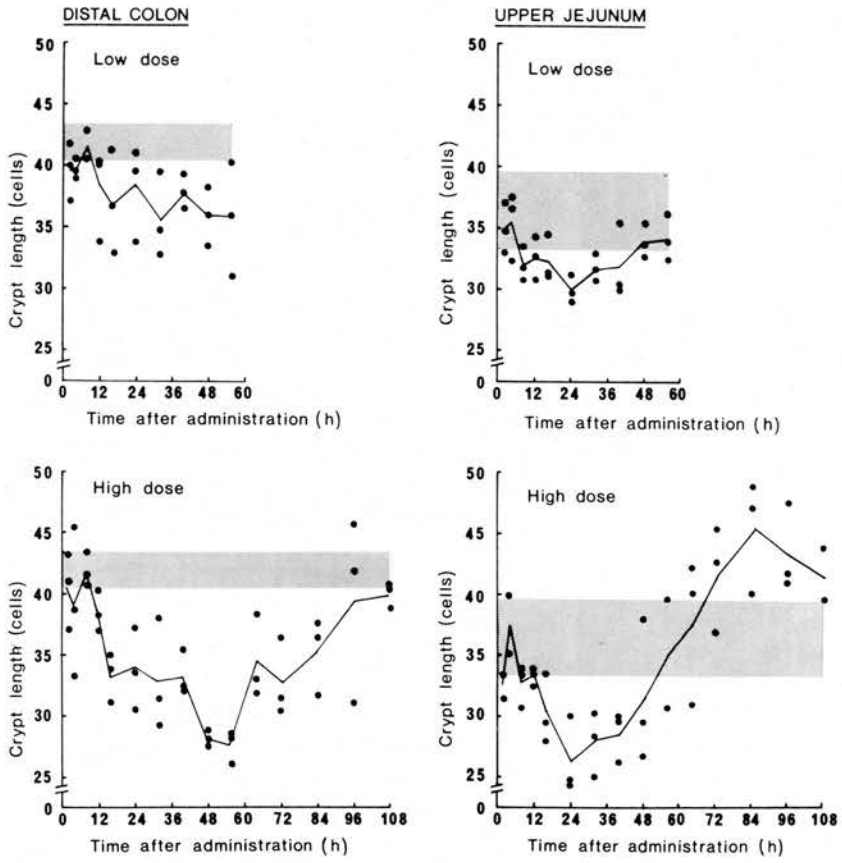

Fig. 5. Mean crypt lengths at various times after DMH in the distal colon and the upper jejunum. The responses to the low dose of DMH are illustrated in the upper panels and to the high doses in the lower. Each point represents the mean of 30 crypts analysed in each section. The shaded area indicates mean ±S.D. of the mean values for the nine control animals

The labelling index (I_s) for the whole crypt epithelium at various times after the administration of DMH is shown in Fig. 7 and 8. No significant changes were evident at two or four hours after DMH and the most profound reductions in I_s were seen at 12 and 16 h. These reductions affected all the sites in the bowel, not just those especially vulnerable to the long term carcinogenic effects of DMH. At 24 h and later the I_s levels were restored to near-normal values. While in the caecum and proximal colon, where pathological loss of cells from the crypts did not occur to any great extent, this may imply a real restoration of the normal proliferative status, the situation is more complicated in the distal colon and upper jejunum. Here extensive crypt cell depopulation has occurred as a result both of toxic damage to cells in the proliferation zone and of normal emigration of cells from the maturation compartment, without effective replacement. These near-normal I_s values should not therefore be taken as evidence of a restoration to normal of the proliferative status,

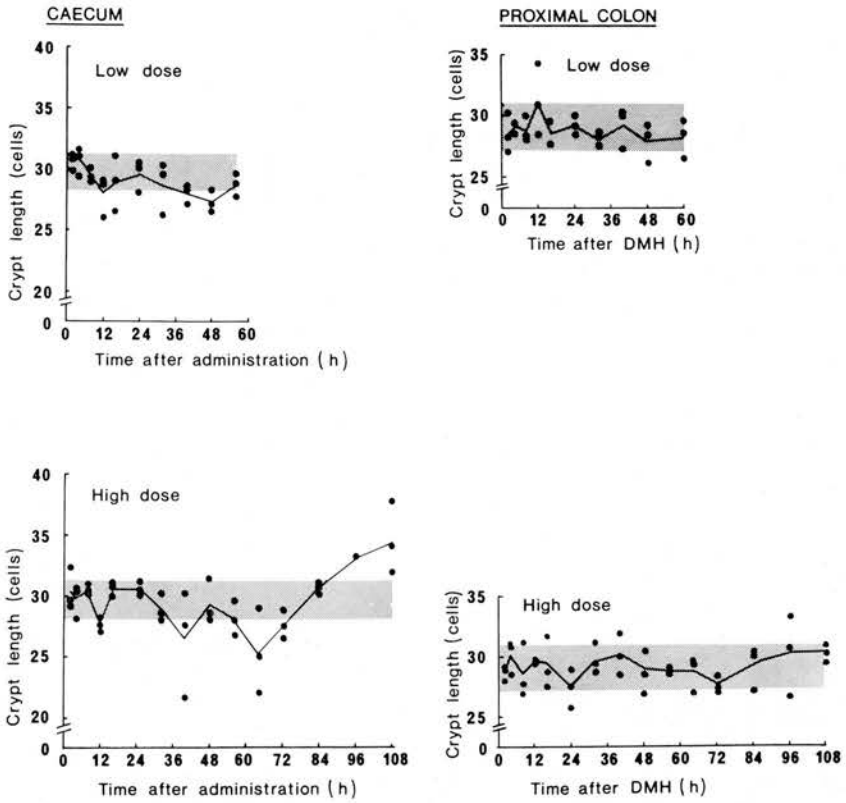

Fig. 6. Mean crypt lengths at various times after DMH in the caecum and the proximal colon. The responses to the low dose of DMH are illustrated in the upper panels and to the high doses in the lower. Each point represents the mean of 30 crypts analysed in each section. The shaded area indicates mean $\pm$S.D. of the mean values for the nine control animals

and on balance they would seem to be more an indication of the relative ineffectiveness of compensatory responses during this phase of continuing crypt cell loss. By 48 h in the upper jejunum, and about 24 h later than this in the distal colon, I_s values reached levels considerably in excess of normal, and these times are a more realistic indication of the stage at which regenerative activity did exceed cell loss (cf. Fig. 5). With the progressive restoration of crypt size, and of a normal villus architecture in the case of the upper jejunum, I_s values began to decline towards normal, a decline that was completed in the case of upper jejunum, but only suggested in the distal colon, by the end of the experimental period.

Both in the caecum and in the proximal colon there occurred an elevation of I_s towards the end of the experimental period with the high dose of DMH. This does not appear to be adequately explicable on the basis of a response to local damage and may well be the result of systemic influences, such as an increased functional demand.

Labelling index distribution diagrams were constructed for each sample taken

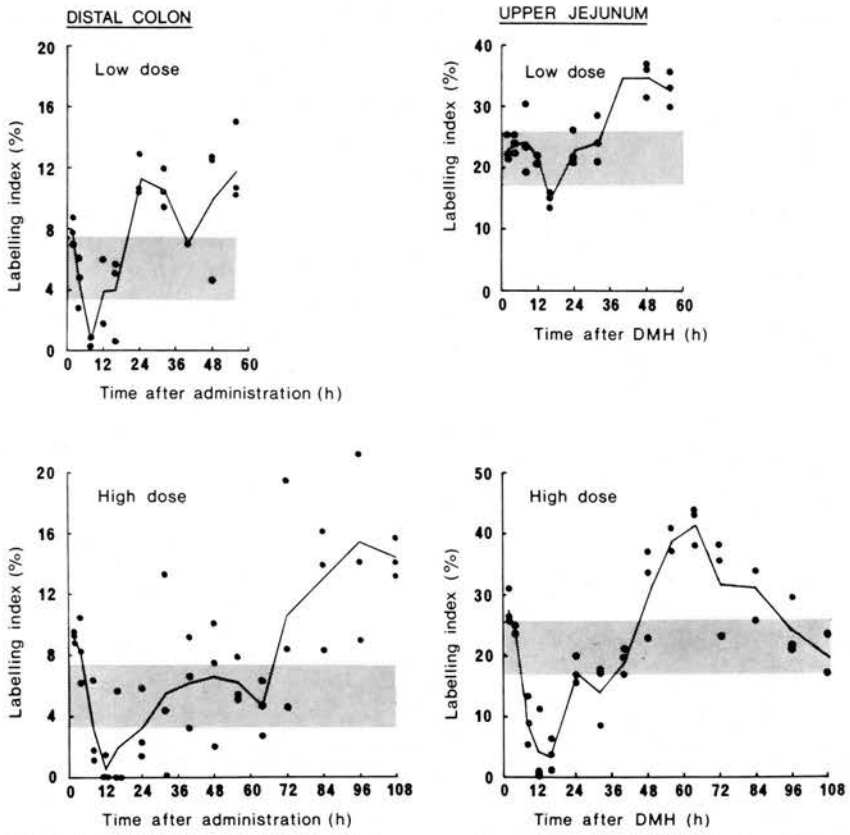

Fig. 7. Mean whole-crypt labelling indices at various times after DMH in the distal colon and the upper jejunum. The responses to the low dose of DMH are illustrated in the upper panels and to the high doses in the lower. Each point represents the mean of 30 crypts analysed in each section. The shaded area indicates mean $\pm$S.D. of the mean values for the nine control animals

from all the experimental animals; it was evident that there were considerable variations even between individual members of a group of three animals killed following the same dose of DMH and at the same time point. However a common pattern did emerge. At all four sites along the length of the bowel I_s was initially depressed in a uniform manner throughout the proliferation zone. When recovery commenced (see Fig. 7 and 8) those sites which normally share the common pattern of a slightly lower I_s in the few cell positions at the base of the crypt than higher up in the proliferation zone (upper jejunum, caecum and distal colon) no longer showed this feature, and for a time in the recovering crypt I_s at the crypt base was essentially the same as that farther up the crypt. Later, when whole crypt I_s had reached levels above normal and when increase in crypt cell numbers was occurring rapidly, the normal I_s distribution pattern was restored, albeit with a temporarily increased proliferation zone. In the proximal colon, where there is a sizeable population of mature

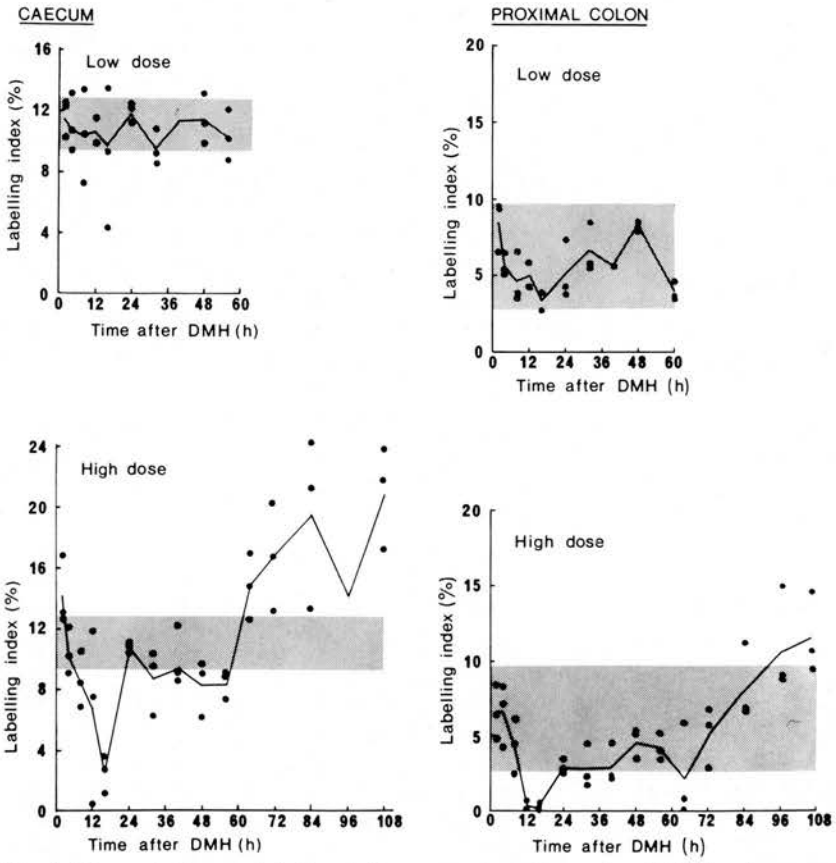

Fig. 8. Mean whole-crypt labelling indices at various times after DMH in the caecum and the proximal colon. The responses to the low dose of DMH are illustrated in the upper panels and to the high doses in the lower. Each point represents the mean of 30 crypts analysed in each section. The shaded area indicates mean $\pm$S.D. of the mean values for the nine control animals

mucus-secreting cells at the base of the crypt in addition to the proliferating cells (Sunter et al. 1979), I_s depression and recovery seemed uniform with no suggestion of the regenerative response occurring earlier at one site within the crypt than at another.

Discussion

The present results confirm that the administration to rats of single sublethal doses of DMH (Druckrey 1970) results in the development of toxic changes in the intestinal mucosae. The sequence of these changes and the subsequent compensatory responses have been documented. The initial destruction of cells in the proliferation zone of the crypt commences within a few hours of DMH injection and continues for several days in the most severely affected portions of the gut; also there is a short lived reduction in the proportion of cells

in DNA synthesis. Following this period of reduced I_s there is evidence of a regenerative response which emerges from a situation of continuing cell damage as shown by the continuing appearance of pyknotic and karyorrhectic nuclei, by other nuclear and cytoplasmic abnormalities and by the presence of abnormal mitotic figures. The regenerative response gains momentum and ultimately results in restoration of the cell-depleted crypts to normal. Similar events have been described by other workers (Loehrs et al. 1969; Hawks et al. 1974), and a similar sequence is seen in the colon of the DMH-treated mouse (Deschner 1978). Following the administration to rats of methylazoxymethanol acetate (MAM), a chemical closely related to DMH, similar changes have also been observed (Zedeck et al. 1970; Zedeck et al. 1977). These workers described differences in the nature and extent of the response in intestinal segments of different vulnerability to the long-term carcinogenic effects of MAM (Zedeck et al. 1977) and in the present study such effects were conspicuous, there being marked perturbations in sites where we have found tumours to be frequent in long term experiments and relatively minor changes in regions where we have seldom observed the late development of tumours (Sunter et al. 1978a, b).

While differences in the long-term incidence of tumours from site to site may be explicable on the basis of differences in the intensity of chronic cocarcinogenic influences, such as the presence of bile acids in the upper small bowel, or bacterial and other constituents in the large bowel, we feel that differences in acute toxic effects are not so explained. They seem to suggest rather a fundamental difference in the susceptibility of the mucosa to the direct effect of the chemical. This is supported by the observations of Gennaro and coworkers (1973), who found that transposition of segments of small intestine to the colon and segments of colon to the small intestine did not alter the susceptibility of the transposed segments to the carcinogenic effects of azoxymethane, a chemical related to DMH. A possible biochemical explantation for this has been suggested by Grab and Zedeck (1977), on the basis of differing concentrations in the mucosa of the enzyme alcohol dehydrogenase, which may play a part in the metabolism of DMH and related compounds.

Acknowledgements. This work was supported by grants from the North of England Council of the Cancer Research Campaign. We wish to thank Miss E. Wark who typed the manuscript, Mrs. E. Wallace who provided invaluable technical assistance and Mr. S. Brabazon and Mr. W. Robinson who prepared the illustrations.

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Received November 11, 1980 / Accepted January 14, 1981

J. Anat. (1984), **139**, 3, pp. 513–523
With 3 figures
Printed in Great Britain

Are there diurnal fluctuations in crypt length and crypt cell birth rate in the intestines of normal and carcinogen-treated rats?*

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(*Accepted* 8 *March* 1984)

INTRODUCTION

Despite early evidence to the contrary (Bertalanffy, 1960; Pilgrim, Erb & Maurer, 1963) it now seems clear that some form of diurnal fluctuation in proliferative indices exists in the intestinal epithelium of rodents. These fluctuations are important in any consideration of cell proliferation in the bowel, implying that cohorts of cells within the proliferating population behave differently at different times of the day.

Most studies of diurnal variation have concentrated on changes in mitotic index (I_m), tritiated thymidine labelling index (I_s) and uptake of labelled thymidine into DNA (Pilgrim *et al.* 1963; Sigdestad, Bauman & Lesher, 1969; Sidgestad & Lesher, 1970; Chang, 1971; Scheving, Burns & Pauly, 1972; Al-Dewachi, Wright, Appleton & Watson, 1976; Potten, Albawari, Humb & Searle, 1977; Scheving, Burns, Pauly & Tsai, 1978; Neal & Potten, 1981). Recently, methods using tritiated thymidine, particularly where labelling is followed by scintillation counting, have been criticised (Maurer, 1981). Greater emphasis has been placed on the measurement of cell birth rate (K_B) and, in the intestine, crypt cell production rate (CCPR) using the stathmokinetic approach. It is claimed that these parameters are more useful indicators of cell proliferation, particularly in comparative studies (Wright, 1980). Bertalanffy (1960) employed the stathmokinetic method in studies of diurnal variation; using colchicine and a long period of metaphase accumulation, he demonstrated the existence of diurnal rhythms in K_B in stratified epithelia but not in columnar epithelium of the intestine. Since this early work there have been few comprehensive attempts to study diurnal changes in K_B in the bowel. Tutton (1975), using colchicine at four time points during the day, has found significant differences in K_B at different times. By contrast, Al-Dewachi *et al.* (1976) chose time points at the zenith and nadir of the diurnal fluctuation in mitotic index which they had documented. They have shown no difference in the rate of metaphase accumulation at these two times following administration of vincristine. Hence, uncertainty still exists as to the extent and significance of diurnal changes in K_B in rodent intestines. Since a considerable proportion of the authors' experiments rely on the calculation of K_B from stathmokinetic data, it was decided to investigate any such changes under *normal animal house* conditions. In the mouse, it has been shown that in both the colon (Chang, 1971; Hamilton, 1980) and the small intestine (Al-Nafussi & Wright, 1982) there are

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significant diurnal fluctuations in crypt size. This has not been documented in the rat, but clearly if present would influence CCPR and affect the interpretation of other kinetic data. Counts of crypt length have therefore been made in parallel with records of mitotic index data.

When administered parenterally to rats, the synthetic carcinogen 1,2-dimethylhydrazine causes the development of epithelial tumours of small and large bowel. These lesions are similar histologically to tumours in man and include both benign and malignant lesions. Tumour development is preceded by a distinct preneoplastic phase characterised by changes in proliferative indices of crypt cells and by mucosal hyperplasia (Sunter, Appleton, Wright & Watson, 1978*a*; Sunter, Watson & Appleton, 1981). It is not known whether this abnormal 'preneoplastic' mucosa shares the diurnal fluctuations exhibited by proliferative indices of normal mucosa. Certainly, when scheduled according to known diurnal rhythms of normal tissues, cytotoxic chemotherapy can enhance tumour cell death in animals bearing transplanted tumours and spare normal tissues (Scheving *et al.* 1977). Therefore, the birth rates of intestinal crypt cells of a cohort of rats treated with carcinogen, kept under identical conditions to normal animals, were also followed over a period of 24 hours, in order to assess any changes in diurnal variation in non-neoplastic but nevertheless kinetically abnormal mucosa.

MATERIALS AND METHODS

Two groups of 48 outbred virgin female Wistar Porton rats were used. The animals were obtained from Olac Ltd, Bicester. Those in the group treated with carcinogen were given a course of 24 subcutaneous injections of 1,2-dimethylhydrazine given at weekly intervals. The course was commenced at 19 weeks of age, when the animals weighed 200–250 g. Each animal received 15 mg/kg of dimethylhydrazine as a freshly prepared solution of dimethylhydrazine dihydrochloride (Aldrich Chemical Co.), 16·6 g/l, in normal saline containing EDTA. The pH of the solution was adjusted to 6·4 using 1 M sodium hydroxide solution. This group of animals was used when they were 47 weeks old, i.e. four weeks after their last injection, to avoid transient perturbations in proliferative activity following treatment. For reasons of expediency the animals in the untreated group were used at 18–21 weeks of age.

Both groups of animals were housed in cages of six and maintained on a normal routine of cleaning and feeding. They were fed on No. 3 expanded rat and mouse breeders' diet (BP) and allowed tap water *ad libitum*. A natural daylight cycle prevailed and on the day of the experiment it was light between approximately 09.00 and 16.30 hours.

On the day of the experiment, commencing at 09.00 hours and at three hourly intervals thereafter, a single cage from each group was withdrawn from the main animal room. From each group, one animal was killed immediately by ether overdose and used for determination of the native mitotic index, while the remaining five animals were given a single intraperitoneal injection of vincristine sulphate (Oncovin, Eli Lilly) at a dosage of 1 mg/kg body weight (Tannock, 1967). These five animals were then transferred to a separate room and kept under the same conditions as in the main animal room. They were killed seriatim at intervals of 30 minutes, the first animal at 30 minutes after the vincristine injection. Forty eight hours elapsed between the experiment on the control animals and that on the treated animals.

All animals were subjected to an immediate postmortem examination, particular

attention being paid to the gastrointestinal tract and any abnormal features. The small intestine was fixed unopened while the large intestine was opened along its length prior to being pinned out on a cork board for fixation. The tissues from the single animal from each cage used for native mitotic index determination were fixed in neutral buffered formol saline solution in order to facilitate histochemical studies. The material from the animals injected with vincristine was fixed in Carnoy's solution for 18–24 hours and then transferred to 2-ethoxy-ethanol.

Following adequate fixation, the material was inspected once again and the size, shape and location of intestinal tumours were noted. Transverse blocks for histological examination were taken from standard sites along the length of the bowel:

(i) proximal small bowel, at a site 20 mm distal to the pylorus, a site known to be at a high risk of tumour development in animals treated with dimethylhydrazine;

(ii) distal ileum, at a site 30 mm proximal to the ileocaecal junction, a site where tumours are infrequently seen;

(iii) proximal colon, 90 % of the distance measured from the anus to the colocaecal junction, a site where tumour incidence is low;

(iv) distal colon, 30 % of the distance from the anus to the colocaecal junction, a site where tumour incidence is high.

These blocks and those from the tumours were processed routinely and embedded in Polywax (Difco). Serial transverse histological sections, 3 μm thick, were cut and stained with haematoxylin and eosin. The slides were randomised prior to assessment to eliminate observer bias. In the carcinogen-treated animals, abnormal crypts lined by epithelium which appeared adenomatous were excluded from the analysis. For each sample, 100 perfect axial sections of crypts were analysed (200 for native mitotic index in colon). The total length of the crypt, from the crypt base (cell position 1) to the crypt/villus or crypt/surface junction was recorded (in cells), as were the cell positions and numbers of mitotic figures or arrested metaphases. The correction factor of Tannock (1967), which compensates for overestimation of mitotic index due to selection of axial crypt sections, was calculated from 200 cross sections of crypts from four animals in the normal and the carcinogen-treated groups for each of the four selected sites in the bowel.

The mean crypt lengths for each group of six animals were calculated and the values plotted against time of day. From the animals in each group accumulation of arrested metaphases was plotted against time after vincristine over the 2·5 hours collection period (Wright & Appleton, 1980) and from the slope of each fitted line the value of K_B and its standard error was calculated. These were then plotted against time of day to assess any patterns of change.

RESULTS

The yield of tumours in animals treated with 1,2-*dimethylhydrazine*

At 28 weeks after the first injection of dimethylhydrazine the incidence of intestinal tumours in the treated animals was lower than that previously experienced in a cohort of animals of the same sex and strain but from a different source, and maintained on a different diet (Sunter *et al.* 1978*a*, *b*). Only a quarter of the animals had large bowel tumours, compared with over 90 % from the earlier investigation and only one of these animals had multiple colonic lesions. While the same range of histological types of tumour was seen as in earlier work there was a relative excess of poorly differentiated carcinomas in the proximal half of the colon. Only 15 % of

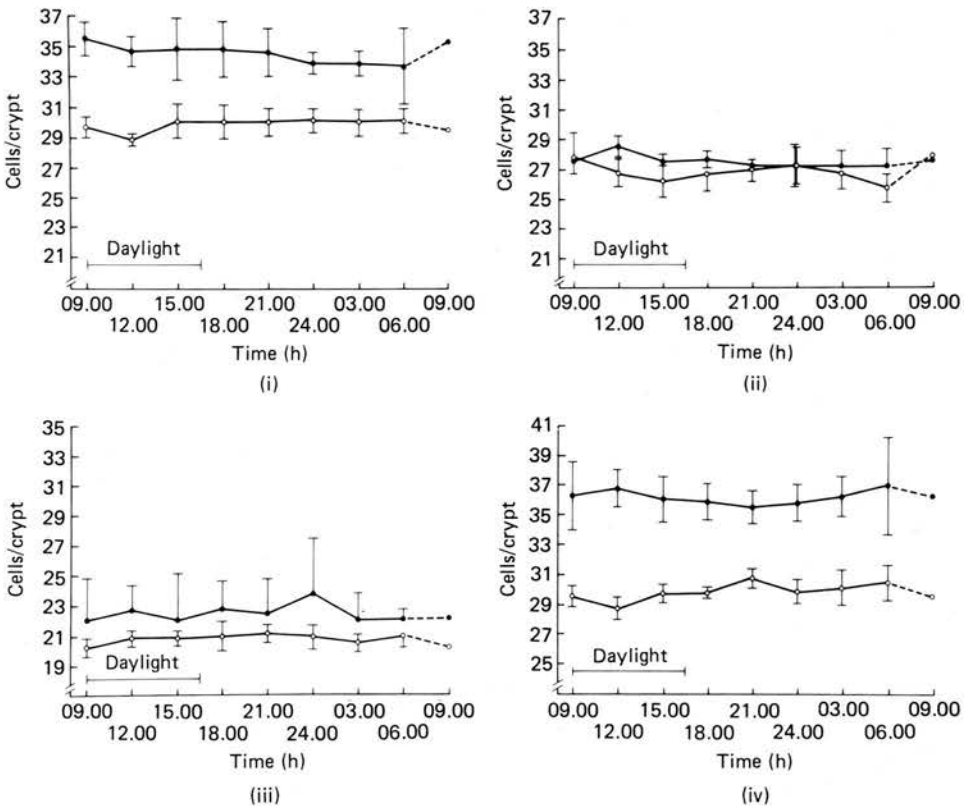

Fig. 1. Crypt lengths (in cells) in control rats and rats treated with carcinogen in (i) proximal small bowel, (ii) distal small bowel, (iii) proximal colon, (iv) distal colon. The values given at each time point are means calculated from the single animal killed for mitotic index determination at that time and the five animals which formed the vincristine study commenced at that time. (i–iii) ○, untreated ±1 S.D.; ●, treated ±1 S.D. (iv) ○, control ±1 S.D.; ●, treated ±1 S.D.

animals in the present investigation had tumours in the proximal 40 mm of small intestine, compared with 37% previously, but again the histological patterns were similar. Five animals had tumours in the region 40–100 mm distal to the pylorus; in contrast with juxtapyloric growths, these were poorly differentiated and tended to be large. No tumours were observed in the distal ileum.

In none of the untreated animals was there any evidence of intestinal neoplasia; this was not surprising in view of the exceptional rarity of spontaneous intestinal neoplasms in rats.

Crypt morphology and crypt cell birth rate

The values of mitotic index obtained from the single animals varied considerably over the 24 hours period, but no overall pattern emerged from either the normal animals or the carcinogen-treated group.

The mean values for crypt lengths over the 24 hours period are presented graphically in Figure 1. At none of the sites in either group was there any significant diurnal fluctuation in crypt length. At the two sites which were at the greatest risk of developing tumours following dimethylhydrazine treatment, the proximal small bowel

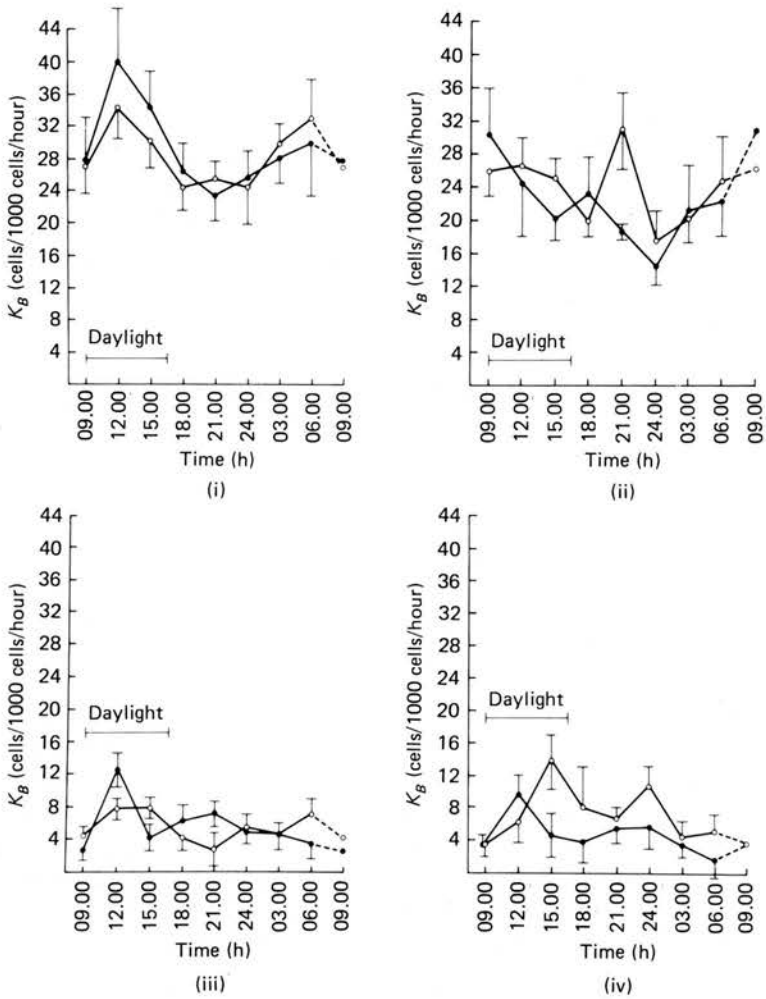

Fig. 2. Crypt cell birth rates (in cells/1000 cells/hour) in control and carcinogen-treated rats in (i) proximal small bowel, (ii) distal small bowel, (iii) proximal colon, (iv) distal colon. The values given at each time point are means calculated from the single animal killed for mitotic index determination at that time and the five animals which formed the vincristine study commenced at that time. ○, untreated; ●, treated.

and the distal colon, there was a significant crypt hyperplasia in the treated animals. In the distal ileum and the proximal colon the difference was much less marked. Variability in crypt lengths in the treated animals was greater than in the controls.

The estimates of birth rate at different times over the 24 hours period are illustrated in Figure 2. The estimates were corrected using calculated values of Tannock's factor (between 0·65 and 0·67) for each site. As shown by the error bars on the illustrations, standard errors were large, and there was no significant difference between normal and treated animals at any time point for any anatomical site. Nor was there any difference in the pattern of change in K_B over the 24 hours period between the two groups. In the proximal small bowel (Fig. 2i) the estimate for K_B in normal animals at 12.00 hours was 35 cells/1000 cells/hour and for animals treated with dimethylhydrazine it was 41 cells/1000 cells/hour. At the same site, K_B

appeared to fall to about 24 cells/1000 cells/hour in both normal and carcinogen-treated animals in the early hours of darkness. Although there appeared to be some sort of circadian periodicity on visual inspection of the data, formal statistical analysis (see Appendix) did not support a real periodic change.

In the distal ileum, K_B estimates ranged from a maximum of 31 cells/1000 cells/hour to 18 cells/1000 cells/hour in untreated animals and 30 cells/1000 cells/hour to 14 cells/1000 cells/hour in treated animals. In the colon, individual estimates of K_B obtained at different times again varied greatly, but there was no consistent pattern of diurnal periodicity. In the distal ileum, proximal colon and distal colon, formal statistical analysis again failed to support the presence of a periodic change.

DISCUSSION

The results confirm previous observations that chronic administration of 1,2-dimethylhydrazine induces a preneoplastic hyperplastic state in the intestinal crypts at sites prone to form tumours in both small and large intestine (Sunter *et al.* 1978*a*, 1981). Hyperplasia occurs despite the fact that in this group of animals mean crypt lengths are smaller in both untreated and treated groups than was previously the case, and that the ultimate yield of tumours is substantially lower. Both differences may reflect intrinsic diversity as the Wistar Porton rats used in the two studies are from different sources and are of different ages; alternatively they may result from a change in diet or intestinal flora. Whatever the reason these substantial differences serve to illustrate the importance of including controls in every kinetic experiment, rather than relying on historical controls or values from other published work.

The principal aims of this study have been to explore diurnal rhythms in crypt length and crypt cell birth rate and to compare normal and dimethylhydrazine-treated rats. A normal animal house routine and a natural daylight cycle were employed. The use of female animals might be seen as adding an extra complication, in the form of the oestrous cycle, in searching for a pattern of change in any proliferative parameters. Exogenous oestrogens certainly alter cell proliferation in the intestine of ovariectomised mice, but only minimal changes have been observed during the natural oestrous cycle (Chang & Hoff, 1980).

The results indicate that neither in normal animals nor in carcinogen-treated rats is there any significant pattern of diurnal fluctuation in crypt length at any of the selected sites in the bowel. These findings contrast with the situation in the mouse where clear diurnal changes, which are in some strains quite remarkable, have been documented (Chang, 1971; Hamilton, 1980).

There has been some criticism of the application of the stathmokinetic technique as a method of detecting diurnal variations (Scheving *et al.* 1972). This mainly relates to earlier studies, but it should be noted that Bertalanffy (1960), using colchicine and a long collection period, was unable to detect diurnal fluctuations in some epithelia. Tutton (1975), using colchicine over a shorter collection period at four time points over 24 hours, has demonstrated a clear pattern of diurnal variation in rats. Thorud and coworkers (1980) concluded that the method is sufficiently sensitive to detect diurnal variation in epidermis. The pitfalls and limitations of the technique have recently been reviewed in detail by Wright and Appleton (1980) and the design of the present study adheres to the recommendations of these authors. The present results show no clear rhythmic patterns of change in crypt cell birth rate in any of the sites selected for analysis when the data are examined statistically, despite the fact that

there is a subjective impression of such a pattern, particularly in the proximal part of the small intestine. Undoubtedly K_B seems to vary a great deal from one time point to another, but there is the impression of a peak during daylight hours at several sites in both groups of animals. It has been suggested that diurnal variation may be an exploitable kinetic factor in optimising cancer chemotherapy (Scheving *et al.* 1977, 1978). In the present study there appears to be no significant difference in the pattern of K_B over the 24 hours period at sites prone to cancer in animals treated with dimethylhydrazine; unfortunately the incidence of tumours was too low to permit a study of diurnal fluctuations in their birth rates.

Most studies of diurnal variation have concentrated on mitotic labelling indices and, with the exception of earlier investigations (Pilgrim *et al.* 1963), have shown peaks and troughs in mitotic and labelling indices with a periodicity of 24 hours. Most of the reports show synchrony of these waves (Sigdestad *et al.* 1969; Sigdestad & Lesher, 1970; Chang, 1971; Scheving *et al.* 1972, 1978; Al-Dewachi *et al.* 1976) although their timings differ. In contrast, several investigations have shown time lags between labelling and mitotic waves (Potten *et al.* 1977; Neal & Potten, 1981; Al-Nafussi & Wright, 1982). The mere existence of peaks and troughs in mitotic or labelling index suggests a degree of synchrony within the proliferative population. Such synchrony would be explicable on the basis of variation in the duration of phases of the cell cycle, the presence of separate, slowly cycling cells within the proliferation zone (Potten *et al.* 1977) and diurnal changes in the growth fraction (Al-Dewachi *et al.* 1976; Al-Naffussi & Wright, 1982). Chalone-like substances have been described in intestinal extracts (Houck, Kanagalingham, Kaufman & Sunshine, 1976; Batt & Scott, 1982) and it is conceivable that these may regulate the flow of cells through G_2 phase. The results of fraction of labelled mitoses experiments do not support anything more than a temporary halt, however, since the first peak of labelled mitoses invariably reaches virtually 100% even at a time of day when the mitotic index is rising. Selective loss of cells from proliferating or functional compartments, if confined to a particular time of day, would produce a diurnal pattern in some proliferative indices. In the light of these several possibilities, interpretation of kinetic data is fraught with difficulties, and it has been argued that comparison of birth rates measured by the stathmokinetic method provides the most useful single index in this sort of situation (Clark, 1973; Wright, 1980). Al-Dewachi *et al.* (1976) selected the zenith and nadir of their diurnal wave of mitotic index as the two points to perform a stathmokinetic study; interestingly they have found no significant differences in the rates of metaphase accumulation in the small bowel of the rat. These results are in keeping with the present findings that while some proliferative parameters may vary periodically the net effects on crypt cell output are less than dramatic and are not convincingly of a periodic nature.

Nevertheless, variations in proliferative indices in some tissues and under some experimental conditions clearly do exist and these variations appear to affect cell birth rates. Their extent and timing also varies with species and strain of animal and it may be important to take these factors into account in the design of some cytokinetic experiments.

SUMMARY

At eight time points over a period of 24 hours, crypt length (in cells) and crypt cell birth rate were measured by the stathmokinetic method with vincristine and evaluated at two sites in the small intestine and two in the colon in Wistar Porton rats. Normal animals were compared with animals which had received 24 injections, at weekly intervals, of the intestinal carcinogen 1,2-dimethylhydrazine. In the treated animals, crypts were significantly longer at sites prone to tumour formation but not at those sites known to be resistant to the effects of the carcinogen. In neither group of animals was there any significant fluctuation in mean crypt length over a 24 hours period. Crypt cell birth rates showed a considerable fluctuation. No difference was noted between normal animals and those treated with dimethylhydrazine and statistical analysis failed to confirm the presence of any true periodic fluctuation.

We would like to thank the Northern Council of the Cancer Research Campaign for financial support, Miss J. Goodwin who provided invaluable technical assistance and Miss A. Lott who typed the manuscript. Also Julian Besag for suggesting the final permutation test.

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APPENDIX: STATISTICAL METHODS

It is not obvious from inspection of Figure 2 that statistical evidence of a diurnal rhythm should be so elusive, especially for the control proximal small intestine, which is reproduced with a fitted curve in Figure 3. Some details are given below of the methods which have been used to analyse the data.

A simple test for non-randomness in a sequence of observations is the non-parametric method, which involves counting how often an observation is either above each of its neighbours or below them (Kendall, 1976). For a cyclic sequence only the maxima need be counted: a small number will indicate positive serial correlation between observations and a large number negative serial correlation. For a set of only eight observations the 5040 different possible sequences are enumerated and the maxima counted in each. A single maximum occurs in 64 cases (1·3 %), two in 1824 cases (36·2 %), 3 in 2880 cases (57·1 %) and 4 in 272 cases (5·4 %). Thus a significant departure from randomness in the direction of a smooth function is detected if there is only one maximum. The observations in Figure 3 display the most frequent number found in random sequences – namely three.

The changes in birth rates may be described by a smooth periodic function such as

$$x = \alpha + (\beta - \alpha) \cos \pi(t - \delta)/12 \quad \text{(Al-Dewachi } et\ al. \text{ 1976).}$$

If the data of Figure 3 are fitted in this way, the curve shown is obtained. It is a poor fit in spite of apparently giving the time of the maximum with a standard error of only 1 hour 40 minutes. Standard errors for curves fitted like this one are heavily model-dependent and may be an order of magnitude too low (Gratton, Appleton & Alwiswasy, 1978). A better method of measuring the performance of the curve is to compare it with what would happen if the observations had been obtained in a different order. It is unnecessary to fit all 5040 possible orderings, but a randomisation test may be applied by generating some of the other situations that might have

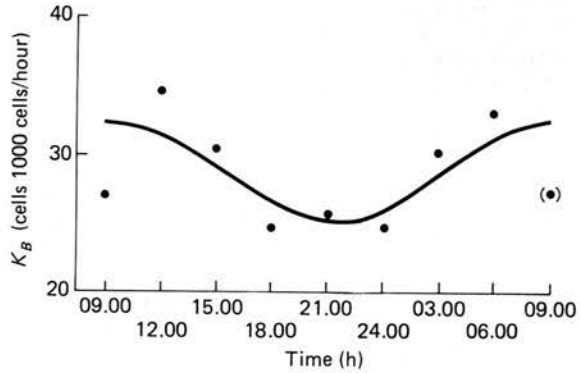

Fig. 3. Crypt cell birth rate (in cells/1000 cells/hour) in the control proximal small bowel with a fitted periodic curve.

Table 1. *Generalised linear interactive model analysis of mitotic index data on control proximal small bowel*

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Linear regression on time during experiment	3247	1	3247	$\gg 1$
Departure from linearity	157	4	39·2	9·3***
Difference between times of day, in mitotic index	108	7	15·4	3·7**
Difference between times of day, in birth rate	17	7	2·4	< 1 NS
Residual	117	28	4·2	
Non-additivity due to imbalance	4			
Total	3650	47		

** $P = < 0{\cdot}01$; *** $P = < 0{\cdot}001$.

arisen, keeping the observations the same and altering only their order. Out of 100 simulations performed, 27 give a residual variance smaller than that of the observed data. Thus once again worthwhile evidence of periodic fluctuation is not detectable.

Neither of the two methods of analysis so far considered uses directly the mitotic indices on which the birth rate estimates were based. Table 1 gives the appropriate analysis of covariance (Snedecor & Cochran, 1967, chapter 14) using proportions, performed using GLIM (generalised linear interactive model; Baker & Nelder, 1978) analysis. There is again no evidence of a diurnal rhythm in birth rate.

Another test is to compare the three daylight readings (09.00, 12.00, 15.00 hours) with the other five using the Mann–Whitney test (Siegel, 1956). This states that if the three daytime readings are higher than the other five readings there would be a significant result at the 5 % level. The three highest observations are not the daytime ones.

Finally, it is possible to take into account what is happening in the treated animals. If the pattern of change in birth rate with time of day is similar, the variance of the eight differences in birth rates should be small compared to what would be obtained if the results were permuted. Of the 40320 different possible permutations, only 164 (0·4 %) give rise to a smaller test statistic, indicating that the patterns – whatever they are – in control and treated proximal small bowel are indeed similar.

In conclusion, the human eye is much more prepared than is statistical analysis to admit to evidence in favour of diurnal rhythm in birth rate. All that can be said is that, whatever the nature of the changes in birth rate in control rats, in the proximal small intestine at least, changes in rats treated with dimethylhydrazine are similar.

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Br. J. Cancer (1984), **49**, 281–290

Morphological studies on the long-term organ culture of colonic mucosa from normal and dimethylhydrazine treated rats

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Summary Mucosal explants were prepared from the colons of normal rats and from the non-neoplastic colonic mucosa of rats which had been treated chronically with the intestinal carcinogen dimethylhydrazine. They were maintained in an organ culture system which permitted survival up to at least 25 days. Morphological preservation of the mucosa was excellent up to 6 days in culture and thereafter changes began to occur. But even at 25 days normal crypt structures were still evident.

The hyperplastic and dysplastic changes seen in pre-culture samples of DMH-treated mucosae remained recognisable during the first two days in culture. They were no longer seen in explants examined after this time however and, indeed, there appeared to be no difference in the morphology and survival of control and DMH-treated mucosae. It is possible that our culture system does not permit further neoplastic progression, but an alternative explanation is that the system discriminates specifically against the survival of neoplastic elements.

The detailed study of cell proliferation in the human intestine *in vivo* is restricted since ethical constraints preclude the experimental use of radioactive isotopes, except under very exceptional circumstances, and limit the application of stathmokinetic agents such as vincristine (Wright *et al.*, 1977). Because of this, short-term organ culture (i.e. for periods up to 48 h), using biopsy or surgical material, has proved useful, and the technique has been widely used to study cell proliferation in both normal and diseased human intestinal mucosa (e.g. Eastwood & Trier, 1973). Biochemical studies have also been facilitated (Hauri *et al.*, 1975; Neutra *et al.*, 1977). Similar short-term organ-culture studies have been carried out on tissues of animal origin (Kedinger *et al.*, 1974; Berteloot *et al.*, 1979; Ferland & Hugon, 1979*a*, *b*). There are however two major difficulties in the interpretation of data from short-term organ-culture experiments. Firstly, it is inevitable that during the procedure of explantation the tissue is subjected to injury of various types such as ischaemia, warming, cooling and mechanical trauma. This may result in a series of immediate adaptive responses which must be allowed for in the selection of an optimum time for an experimental procedure (Pritchett *et al.*, 1982). Secondly, in most culture systems for intestine there occur further profound changes in cell proliferation after 48 h (Johnson, 1970; Senior *et al.*, 1982). These two factors therefore limit the type of kinetic experiment which can be performed. Clearly, therefore, it is desirable to prolong that period in organ culture where structure and function are well maintained and hopefully are representative of what happens *in vivo*.

Acting on the hypothesis that normal collagen metabolism is essential to the survival of tissues consisting of a mixture of epithelial and connective tissue elements, Hodges & Melcher (1976) developed a defined medium, containing ferrous ions and ascorbic acid, which permitted the prolonged maintenance in culture of foetal mouse mandible. They found also that the addition of hydrocortisone improved survival, and ascribed this to an inhibition of the release of lysosomal enzymes. Defries & Franks (1977) successfully used this medium, supplemented by foetal calf serum (FCS), to culture adult mouse intestine, albeit with some morphological change. Recently we have applied this system to the culture of human colonic mucosa obtained from surgical resection specimens (Senior *et al.*, 1982) and have found that this tissue undergoes a remarkable phase of regenerative activity following a degenerative phase which occurs after 2–4 days in culture. Using this system we have maintained explants for up to 23 days; as yet we have not made observations beyond this period.

In the present report we describe further modifications of this culture system, designed to facilitate the prolonged survival of the intestinal mucosa of rats. If such a system were good enough

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Received 21 November 1983; accepted 14 December 1983.

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it would provide a useful means of investigating the effects of various putative trophic agents on the intestine, since in culture the direct effects of these agents would be seen in the absence of the compensatory phenomena which cloud the interpretation of *in vivo* experiments. The direct effects of drugs and toxins could also be monitored and, an aspect of particular interest, new light could be shed on the process of intestinal carcinogenesis.

The synthetic chemical carcinogen 1,2-dimethylhydrazine (DMH), when administered parenterally to rats, causes the development of multiple carcinomas of both small and large intestine (Druckrey *et al.*, 1967; Druckrey, 1970; Sunter *et al.*, 1978*a*, *b*). The period of latency prior to the development of tumours varies with the dosage schedule of the drug and the strain of animal used. In the system we have employed we have observed immediately prior to the development of frank neoplasms a state of mucosal hyperplasia in both the small intestine (Sunter *et al.*, 1978*a*) and in the colon (Sunter *et al.*, 1981). This hyperplasia is confined to tumour-prone sites in the bowel and is accompanied by kinetic changes which have been documented (Sunter *et al.*, 1978*a*, 1981). The changes appear stable and are readily reproducible. In view of this it was considered of interest to examine how the abnormal mucosa compared with the normal in its adaptation to and survival in the *in vitro* environment. If the hyperplasia and other changes do represent a specific preneoplastic state then some morphological expression of this might be expected in culture. On the other hand if further carcinogenic influences are needed to produce frank neoplasia such changes may not be seen. To investigate these possibilities we have therefore compared the performance in long-term culture of explants of colonic mucosa taken from normal rats with that of colonic mucosal explants from rats treated chronically with DMH.

Materials and methods

Animals, treatment and culture techniques

Virgin female albino Wistar Porton rats were used throughout. The animals were obtained at an age of eleven weeks from Olac Ltd., Bicester, and were allowed a four week settling-in period. Following this the animals in the DMH treatment group began a 24 week course of carcinogen injections. The DMH was administered weekly by the subcutaneous route at a dosage of $15\,mg\,kg^{-1}$ body weight of base as a solution consisting of 1.66 g per 100 ml of dimethylhydrazine dihydrochloride (Aldrich Chemical Co., Milwaukee, Wisconsin). The DMH was dissolved in normal saline containing 1.5% EDTA, and the solution adjusted to pH 6.4 by the addition of 1 M sodium hydroxide solution. It was freshly prepared each week. Both groups of animals were maintained under normal animal house conditions; they were fed on Rat and Mouse Breeders Diet (Special Diet Services) and allowed tap water *ad libitum*.

At various times after the final DMH injection (v.i.) groups of DMH-treated animals and control animals of the same age were killed by cervical dislocation. Immediately *post mortem* the whole intestinal tract was removed and the large intestine opened along its length and emptied of faeces. A segment of colon 100–120 mm in length, and ending 40 mm from the anal margin, was removed and gently washed in cold Hanks' balanced salt solution containing $100\,U\,ml^{-1}$ penicillin, $100\,U\,ml^{-1}$ streptomycin and $100\,U\,ml^{-1}$ mycostatin (Gibco). The small intestine and that part of the colon not placed in Hanks' solution was then carefully examined. The appearance and location of intestinal tumours were noted and blocks of them were taken for routine microscopy. General *post mortem* findings were also recorded and tissue blocks were taken from any metastatic deposits or other pathological lesions.

The isolated segment of intestine was then subjected to stereo-microscopic examination. Using fine watchmakers' forceps the mucosa and muscularis mucosae were stripped from the underlying muscularis propria in the plane of the submucosa. This was most easily accomplished by first detaching one corner of the tissue and working from the proximal end to the distal. The mucosa was then spread, luminal surface uppermost, on a sheet of sterile dental wax. The presence and nature of any tumours were noted and they were excised with 3–5 mm of adjacent mucosa. The rest of the mucosa was divided into a number of explants each measuring about 3×5 mm and these were transferred to the support phase, which consisted either of cellulose acetate filters or triacetate filters (Gelman).

The culture medium for maintenance of the explants consisted of Waymouth MB 752/1 (Gibco) containing $100\,U\,ml^{-1}$ each of penicillin, streptomycin and mycostatin (Gibco) supplemented with 10% foetal calf serum (Northumbria Biologicals), $300\,\mu g\,ml^{-1}$ ascorbic acid (Sigma), $3\,\mu g\,ml^{-1}$ hydrocortisone – 21 – sodium succinate (Sigma) and $0.45\,\mu g\,ml^{-1}$ ferrous sulphate (BDH). Cultures were maintained at 37°C in an atmosphere of 95% O_2 and 5% CO_2 within a humidified controlled atmosphere chamber (Bellco) rocked at 8 cycles min^{-1} on a rocking platform (Bellco) so that the medium was washed over the explants intermittently.

Those explants selected for morphological evaluation by light microscopy were fixed in 10% neutral buffered formol saline and then processed routinely into paraffin wax (Fibrowax, 50% and Pure Paraffin wax 50%, Lamb). Vertical 3 μm sections were cut through each block and stained with Harris's haematoxylin and eosin. Representative sections were also stained by the PAS method, Gordon and Sweet's reticulin method and by Miller's elastic-van Gieson. Blocks for electron microscopy were fixed in 2% gluteraldehyde for 1–1.5 h, stored in cacodylate-sucrose buffer and post-fixed in osmium tetroxide. The tissue was epoxy-embedded, ultrathin sections were cut and double stained with uranyl acetate and lead citrate. Low power microscopy was undertaken in order to assess cellular changes.

Experiment 1

Two groups of control and two of treated rats were used. The first group was used to set up cultures 4 weeks after the treated animals had been given their final DMH injection, and the second group 8 weeks after the final injection. In both cases eight treated and six control animals were used. In the first experiment a support phase of 3 μm pore size cellulose acetate filter membrane (Millipore) was used. The cultures were maintained in 100 mm square petri dishes (Sterilin) to which 10 ml of medium was added. Material from 2 animals of the same group was accommodated per dish. The medium was changed after 24 h and thereafter at 2-day intervals. A representative sample of colonic primary tumours was also cultured. Preculture samples (Day 0) from each animal were obtained and also a minimum of four explants for light microscopy and two for electron microscopy on Days 1, 10 and 20.

Experiment 2

In order to study the adaptive changes during the early part of long-term culture a second batch of animals was used, and samples were fixed at 0, 1, 2, 3, 4, 5, 6, 10, 15, 20 and 25 days. Clearly only a limited amount of tissue is provided by a single animal and although 17 control and 17 treated animals were used it was necessary to devise a sampling strategy in order to permit a number of replicate samples per animal at any one time point. Thus the material from each animal provided observations at 4 time points (in addition to times 0 and 1) and at each time point material from 6 control and 6 treated animals was obtained.

Cultures were set up as before on Gelman GA4 triacetate membranes in 60 mm vented petri dishes (Sterilin) in 2.5 ml of medium. Because of the smaller volume of medium involved it was changed daily throughout the experiment. Three or 4 explants per animal were fixed at each time point for light microscopy and explants from at least 2 animals per time point were fixed for electron microscopy.

Results

In none of the control animals was there any evidence of the formation of intestinal tumours and this is to be expected in view of the exceptional rarity of spontaneous intestinal tumours in laboratory rodents. The incidences of small intestinal and colonic tumours in the DMH-treated animals were somewhat lower than we have observed previously in animals of the same strain obtained from a different source (Sunter *et al.*, 1978*a, b*). Of 25 rats killed 4 weeks after the end of DMH treatment 12 animals had large bowel tumours, which were multiple in 5; eleven animals had small bowel tumours. Three animals showed metastatic dissemination of intestinal carcinomas. Of the 8 animals killed 8 weeks after the end of treatment 5 had large bowel tumours. The types and distributions of tumour were broadly similar to those previously observed. Despite the reduced tumour incidence there was generalised crypt hyperplasia in the treated animals and dysplastic crypts were identifiable quite frequently in preculture samples (Figure 1). These latter changes were more conspicuous in the animals killed 8 weeks after cessation of DMH treatment.

In the first experiment none of the cultures was lost to infection. In the second experiment 4/51 plates from treated animals and 13/51 plates from control animals became contaminated. Since in several cases not all the samples from one animal were contaminated it was concluded that this problem was not a failure to suppress enteric microflora but rather contamination from an exogenous source.

Virtually no difference was observed between the behaviour of control mucosa in culture and that of treated mucosa. The results of Experiments 1 and 2 will be considered together. The separation of the mucosa from subadjacent muscularis propria was in most cases achieved with relatively little traumatisation. When significant trauma did occur however it was easily detected microscopically in time zero material. After 24 h in culture adaptive changes were obvious (Figure 2). Crypt cells showed a considerable loss of intracytoplasmic mucin, resulting in increased cytoplasmic basophilia. Basic crypt architecture was well maintained however and proliferating cells remained normally distributed as evidenced by the

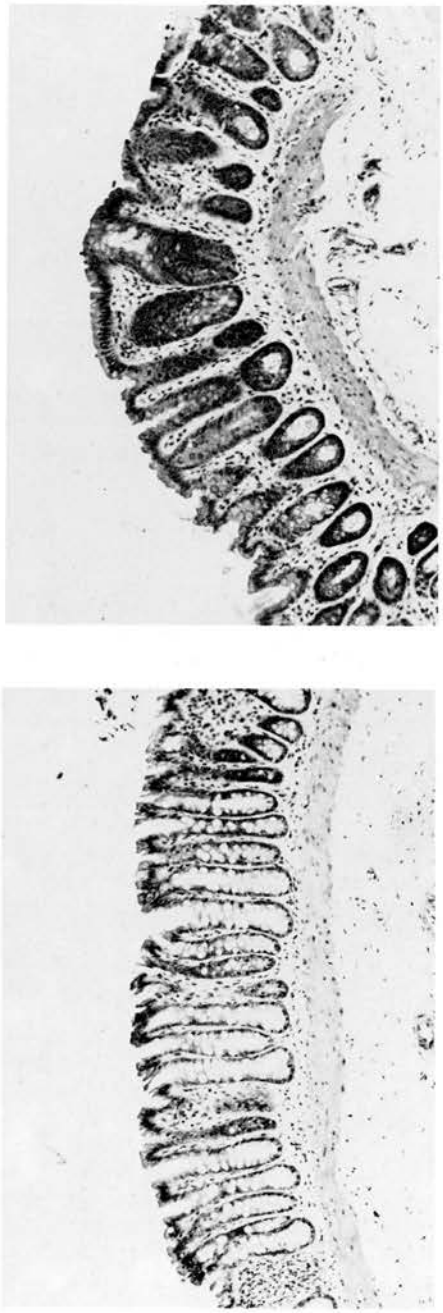

Figure 1 Dissected samples of mucosa prior to culture (a) control (b) DMH-treated. In the treated material some crypt hyperplasia is evident and there are focal dysplastic changes in some crypts. (H & E × 225).

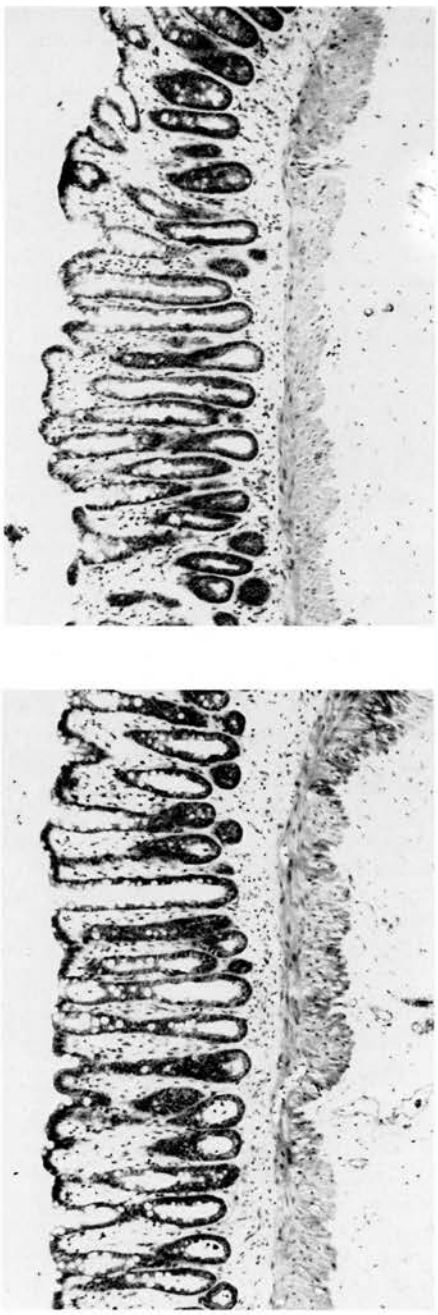

Figure 2 Explants of (a) control and (b) DMH-treated mucosae after one day in culture. Similar changes have occurred in the two mucosae. Some dysplastic crypts are still seen in the treated mucosae. (H & E × 225).

limitation of mitotic figures to the lower two-thirds of the crypt. The overall impression gained was that the crypts were more "spread out" than usual. This was at least in part accounted for by the marked reduction in the population of mononuclear cells in the lamina propria, which occurred even at this early stage of culture. No other alteration was apparent in the lamina propria. Overall the preservation of both control and treated mucosae was excellent. But areas of trauma sustained during dissection were clearly visible as discrete areas of complete crypt loss and lamina propria depopulation. It was possible to discern in some DMH-treated mucosae residual evidence of hyperplasia, and occasional dysplastic-looking crypts, but these differences were less conspicuous than they had been in preculture samples.

During the next 5 days in culture no evidence was seen of the degeneration and regeneration phases which occur in human colonic mucosal explants (Senior *et al.*, 1982). Instead relatively minor changes were apparent, consisting of a further reduction of epithelial-cell mucin, slight crypt shortening and some collapse of the lamina propria. Mitotic figures distributed as normal were seen in approximately normal numbers within the crypts, but not at the surface. Control mucosae and DMH-treated mucosae were indistinguishable (Figure 3) and, after day two dysplastic crypts were no longer identifiable in the latter. Central necrosis in the explants was seen only when the size of the explants exceeded 3×5 mm. By 10 days more profound changes were apparent: most of the explants were much reduced in size, the lamina propria being collapsed, acellular and hyalinised. Some crypts retained a near-normal form (Figure 4) with a relatively normal distribution of mitoses, but were reduced in numbers and shortened. Others were distorted to form tiny cyst-like formations or structures of irregular shape often grouped together. The epithelium lining these abnormal structures appeared crowded and hyperchromatic and had a vaguely adenomatous appearance, reminiscent of the dysplastic crypts seen in preculture DMH-treated mucosa. These changes were seen however in both control and treated material with a roughly equal frequency, and are illustrated in Figure 5. This mixed appearance persisted for the rest of the culture period; even at 25 days well formed crypts remained in some material (Figure 6). At the electron microscopic level (Figure 7) cellular polarity was preserved and microvilli were still evident at the luminal border. The basement membrane complex was intact, but there were large intercellular spaces with interdigitating processes, a feature which has been commented upon previously in organ culture material (Defries & Franks, 1977). Tight junctions were still evident between the cells near their apices.

The mixed appearance of the explants, with normal and abnormal crypt structures, persisted over the rest of the experimental period up to 25 days. Even at this time well differentiated crypts with goblet cells were preserved. In areas of explants devoid of crypt structures the surface epithelium remained intact, and occasionally contained mitotic figures.

Attempts to culture the tumours from the DMH-treated animals met with little success. Of the nine tumours cultured all showed very extensive central necrosis occurring in the earliest stages, with survival of only a narrow rim of tumour tissue at the surface of the explant.

Discussion

A number of systems have been proposed for the prolonged culture of adult rodent mucosa. Schiff & Moore (1980) used a medium similar in composition to that presently described to culture adult rat colonic mucosa. They used a static organ culture system with a support phase consisting of human fibrin foam; survival was observed up to 21 days. In our own initial studies we used a static technique closely similar to theirs and the same as that which we employed for human colonic mucosa (Senior *et al.*, 1982), but had little success. Use of a rocking technique, coupled with medium supplementation with steroid hormones has greatly improved both survival and the degree of mucosal preservation. These measures have been used by several other groups to gain good preservation of rodent mucosa in different media formulations (Autrup *et al.*, 1978; Shamsuddin *et al.*, 1978; Reiss & Williams, 1979), and in general the tissue preservation obtained by these groups has been similar to that seen in the present study despite the differences in media. Autrup *et al.* (1978) originally maintained their explants at 30°C which might be a disadvantage in any functional studies. A further observation is that several of these groups had success using full-thickness explants of bowel wall; we found in pilot studies that the survival of full thickness explants was less than satisfactory.

In common with the observations of Defries & Franks (1977), who used mouse tissue, we found no difference in the adaptive responses to culture between control mucosa and DMH-pretreated mucosa. Nor did we observe any difference in culture between treated mucosa explanted 4 weeks after the end of DMH treatment and that explanted 8 weeks after. This was despite the presence of clear hyperplastic and dysplastic changes observable in the treated mucosa prior to culture.

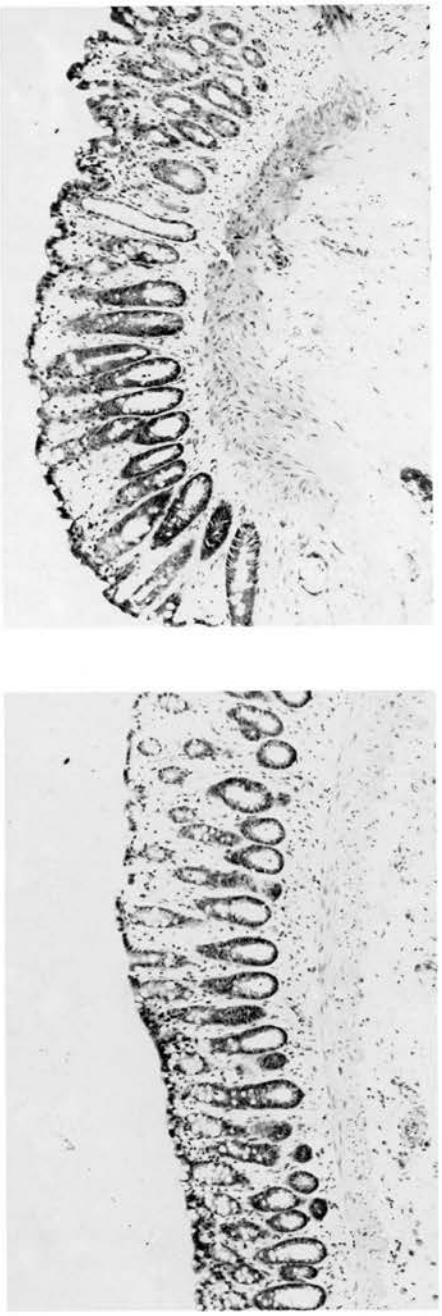

Figure 3 Explants of (a) control and (b) DMH-treated mucosae after 4 days in culture. The appearances are now virtually indistinguishable. (H & E × 225).

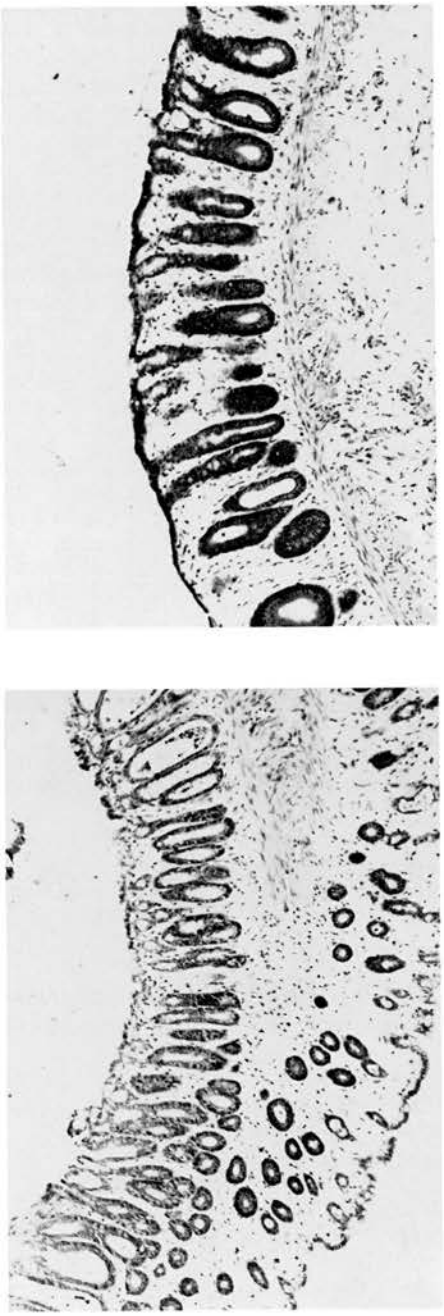

Figure 4 Explants of (a) control (H & E × 175) and (b) treated mucosa (H & E × 225) after 10 days in culture. In the control mucosa some folding of the explant has occurred, but the mucosae are indistinguishable.

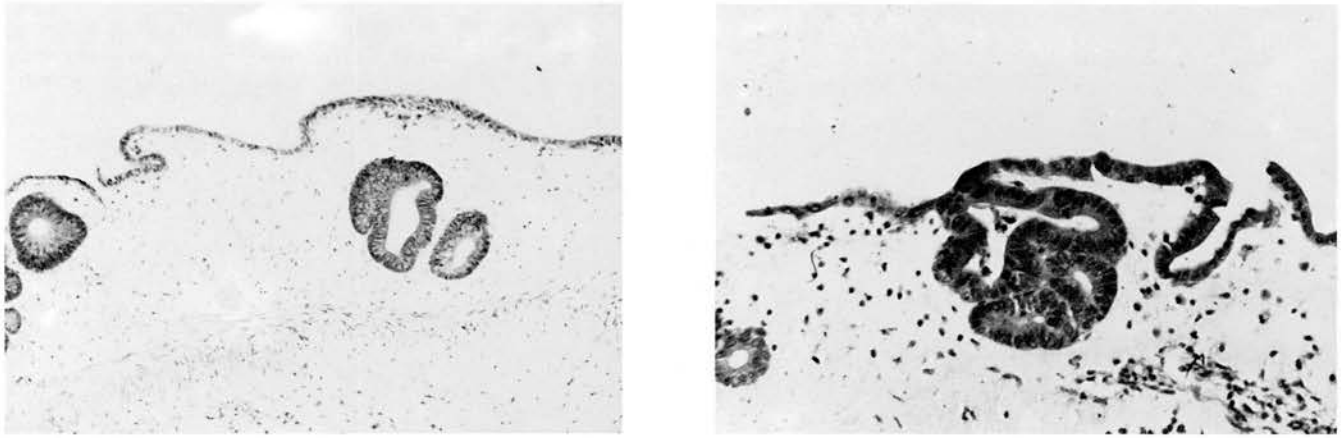

Figure 5 Explants of (a) control (H & E × 225) and (b) treated mucosae (H & E × 325) after 10 days, showing vaguely adenomatous formations.

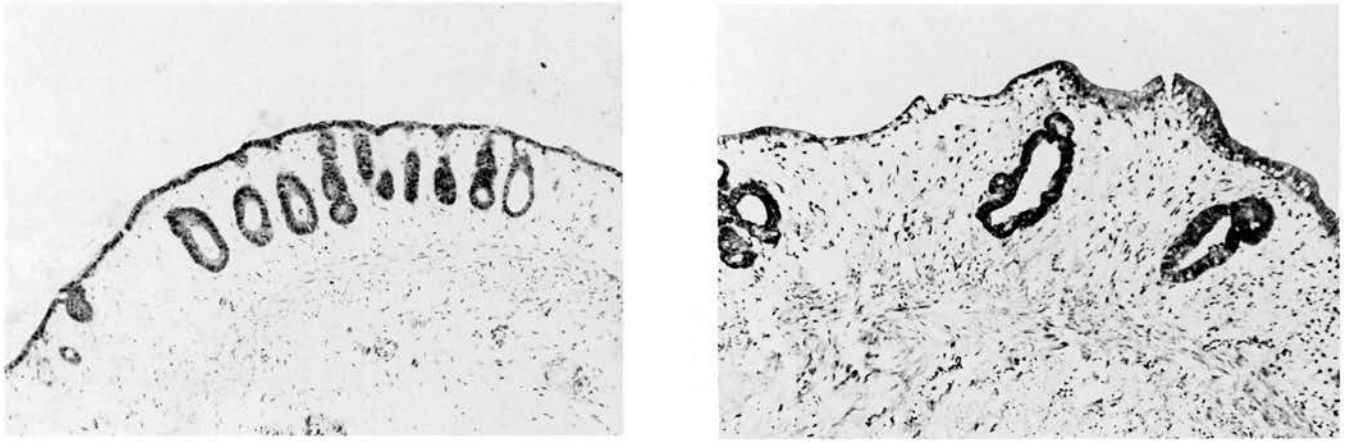

Figure 6 Explants of (a) control (H & E × 225) and (b) treated mucosae (H & E × 325) after 25 days in culture. Some well-formed crypt structures are still apparent in both (H & E × 325).

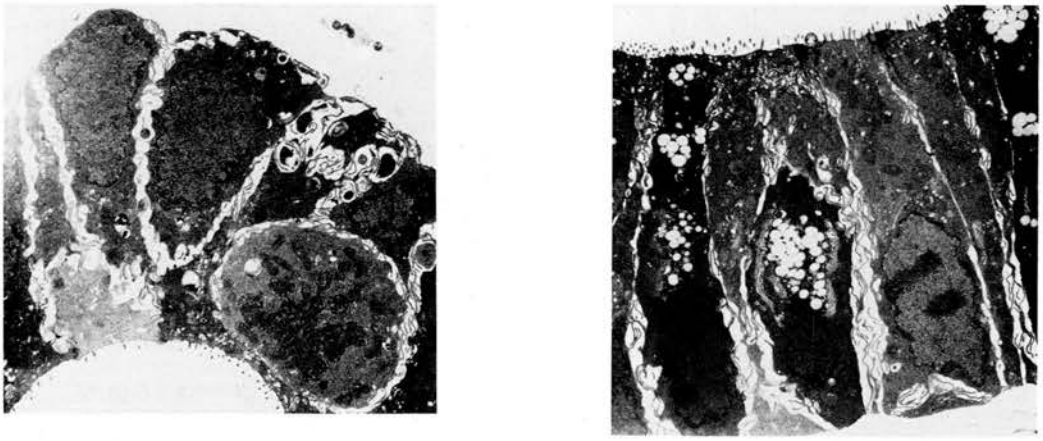

Figure 7 Control (a) and treated (b) mucosa after 10 days in culture. Cellular polarity if preserved but large intercellular spaces have developed.

Maskens (1982) has suggested that DMH carcinogenesis in the rodent is a two-stage process. The first stage, which could be seen as corresponding to the initiation phase of skin carcinogenesis (Friedewald & Rouse, 1944), results in a stable, heritable change in a proportion of cells, which does not confer upon them any advantage over those not so affected. So there is no build up of these cells within the tissue nor any tumefaction. The relatively minor hyperplastic and dysplastic-type changes seen in the non-neoplastic mucosae of DMH-treated animals might well reflect such a change. The second, or promotion, stage occurs later and does not appear to require the presence of the carcinogen since even a single dose of DMH can effect the production of tumours after a latent interval of months (Schiller *et al.*, 1980). It ends in the appearance of frank neoplastic disease. Such a two-stage process has been postulated on the basis of proliferative defects appearing during the genesis of human colonic cancer (Lipkin, 1974), and recently the conventional polyp-cancer theory of human colonic neoplasia (Morson & Dawson, 1979) has been modified to accommodate a dysplasia-neoplasia progression (Konishi & Morson, 1982).

The precise mechanism by which DMH induces intestinal neoplasms is still obscure, but it has been shown to affect DNA synthesis in colonic epithelium in organ culture (Mak & Chang, 1978; Reiss & Williams, 1978; Telang *et al.*, 1980) and to bind to nuclear DNA in such cells (Autrup *et al.*, 1978). Prolonged exposure to DMH *in vitro* results in enhanced tritiated thymidine labelling indices, although it is not known if these changes persist when DMH is removed from the culture environment (Telang & Williams, 1982). *In vivo*, different segments of the intestine show differing susceptibilities to DMH carcinogenesis (Sunter *et al.*, 1978*a*, *b*), and to the acute toxic effects of the chemical (Sunter *et al.*, 1981). Thus either the carcinogenic stimulus, or indeed carcinogenic agents such as those discussed by Hill (1975) in the context of human cancer, is available only in these susceptible sites, or the epithelia at these sites have some inherent predisposition. Transposition of segments of tumour-susceptible and tumour-resistant bowel does not, however, alter their vulnerability to tumour formation (Gennaro *et al.*, 1973) and exclusion of segments of bowel from the faecal stream did not prevent tumour formation in the by-passed region (Wittig *et al.*, 1971; Rubio *et al.*, 1980; Rubio & Nylander, 1981). These observations strongly support the notion that DMH and any promoting factors are acting systematically on inherently susceptible cell populations.

The DMH-treated mucosa used in this study has undergone a stable change in its proliferative characteristics (Sunter *et al.*, 1981) and this is likely to be the result of initiation of the neoplastic process. But *in vitro* the final stimulus to neoplastic transformation may well be lacking, since no example of this was seen. A major problem with this suggestion is that the dysplastic crypts, readily apparent in preculture DMH-treated samples disappeared in the cultured samples after as little as forty-eight hours. While the small kinetic and morphometric differences between normal and DMH treated crypts could easily be masked by the profound adaptive changes occurring during the early stages of culture, it is more difficult to accept

that dysplastic changes could be so easily masked. It is tempting to suggest that the culture conditions do not favour the survival of preneoplastic crypts and that they are selectively lost during culture. And in this context it may be significant that the established tumours from the treated animals fared very poorly in culture. Further studies are under way to compare the adaptive kinetic responses of normal and DMH-treated mucosae to the culture system. Work is necessary to determine whether further applications *in vitro* of DMH or other stimulants of cell proliferation can effect neoplastic transformation of mucosae in this culture system.

This work was supported by a grant from the Northern Council of the Cancer Research Campaign. We would like to thank Miss A. Lott who typed the manuscript, the technical staff of the histopathology laboratory in the Queen Elizabeth Hospital who prepared the material for light microscopy, Mr. T. Coaker for electron microscopy and Mr. S. Brabazon who prepared the illustrations.

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David Appleton

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Demonstration of vincristine resistance in primary intestinal neoplasms in the rat by the 'Post-metaphase Index'

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Summary A method is described enabling the direct measurement of vincristine resistance in intact tissues *in vivo* by morphological study. Using the metaphase arresting properties of the drug, counts were made of escaping anaphase and telophase mitotic figures at a range of doses. The proportion of post-metaphase mitotic figures is called the post-metaphase index (PMI). In 95 primary intestinal tumours induced by dimethylhydrazine (DMH) in rats, an increase in resistance to vincristine was shown over normal mucosa ($P<0.001$). The data were analysed by computer modelling and a linear relationship is demonstrated between the logit of the post-metaphase index, and log dose of vincristine. To achieve a PMI of 1% the fitted lines show an enhanced vincristine dose requirement over normal mucosa of 6 times in colonic tumours, and 8 times in small intestinal tumours. Non-neoplastic mucosa from the DMH-treated animals requires an enhanced dose of vincristine of 1.5 times, compared with normal mucosa, to achieve a PMI of 1%.

Given current interest in the mechanism of vincristine resistance in cell lines this new approach provides a technique for assessing the resistance of solid tumours, both *in vivo* and *in vitro*, and for subsequent experimental manipulation.

Certain drugs, including the Vinca alkaloids and colcemid, have the ability to arrest dividing cells during the mitotic process, by inhibition of microtubular polymerisation in the formation of the mitotic spindle. The resulting 'arrested metaphase' mitotic figures are readily recognisable and provide one approach to the determination of indices of cell proliferation by morphological means, the so-called stathmokinetic experiment. In conventional cell-kinetic studies a large dose of the stathmokinetic agent is used in order to arrest all cells entering mitosis, and at full arrest no mitotic figures appear as anaphases or telophases. The rate of accumulation of the dividing cells then gives the cell birth rate (Tannock, 1967; Steel, 1977).

In a recent stathmokinetic study of human colorectal carcinoma grown in organ culture Pritchett *et al.* (1982) showed that tumours required a sixfold larger dose of vincristine to achieve full metaphase arrest than did non-neoplastic mucosa from the same resection specimen. This *in vitro* observation mirrors the disappointing clinical experience of vincristine treatment of bowel cancer. Much current interest relates to the biochemical, pharmacological and genetic basis of the observed resistance of cancer cells to chemotherapeutic agents. In the case of resistance to the Vinca alkaloids there is an association with the pleiotropic multidrug resistance phenotype (Ling *et al.*, 1983) involving the cell surface glycoprotein P180 (Garman *et al.*, 1983). In several studies using cell lines of both rodent and human origin, in which either inherent or induced resistance was manifest, it has been shown that a variety of calcium transport antagonists, and other drugs will abolish resistance (Tsuruo *et al.*, 1982; 1983*a*; 1983*b*; Ramu *et al.*, 1984). This increase in sensitivity is accompanied by increased intracellular accumulation of the drug.

It is clearly of importance to understand more about vincristine resistance and to document it more fully in human and *in vivo* systems. The normal stathmokinetic approach, as adopted by Pritchett *et al.* (1982), is designed to maximise the accuracy of estimations of cell birth rate. It is both inappropriate and poorly adaptable to a dose-response format, and we have therefore developed a method of assessing the sensitivity of tissues to vincristine not by comparing doses required for complete arrest, but by directly assessing escape from metaphase arrest at various doses. In the present study we have examined the phenomenon of vincristine resistance in dimethylhydrazine (DMH) induced primary epithelial tumours of the intestine in rats. This is the first documented direct characterisation of vincristine resistance in primary solid tumours *in vivo*.

Materials and methods

Rats and treatment schedules

Ninety-eight male Wistar rats (Olac Ltd, Bicester) were used. They were maintained in standard conditions throughout the experiment with unrestricted access to food (Breeders diet no. 3, Special Diet Services Ltd, Witham) and tap water.

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Received 18 March 1985; and in revised form, 1 July 1985.

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The animals were divided, at between 10 and 11 weeks of age, into control (30 animals) and DMH-treated (68 animals) groups. The DMH-treated animals received a long-term, low-dose schedule of DMH (Aldrich Chemical Co. Ltd, Dorset) exposure, comprising 24 subcutaneous injections, administered at one week intervals, of a dose of 20 mg (of base) kg^{-1} body weight. Animals were killed at least two weeks after the final dose of DMH to allow recovery from any acute toxic effects.

Vincristine experiment

The animals of both treated and untreated groups were allocated at random into six vincristine dosage groups (Oncovin, Eli Lily Ltd, Basingstoke) ranging from 1.0 mg kg^{-1} body weight, a full metaphase arresting dose for rat colonic epithelial cells *in vivo*, to 0.01 mg kg^{-1} body weight. Vincristine was administered during a period in which the rats were between 38 and 45 weeks old, an age at which previous experience had shown that tumours are frequent, while morbidity due to tumour effects is still low. The control animals were divided equally into six groups of 5 animals. The DMH-treated animals were divided into two groups of 12 animals (for doses 1.0 mg kg^{-1} and 0.01 mg kg^{-1}) and four groups of 11 animals. Vincristine was administered as a single i.p. injection given between 0900 h and 1100 h to minimise effects of diurnal variation. All animals were killed 2 h after vincristine administration by cervical dislocation, and autopsy was performed immediately. The small bowel was dissected free and fixed unopened for at least 10 h in Carnoy's fluid. The colon was dissected free with a margin of anal skin, opened along its length, and pinned to a cork board prior to Carnoy fixation. The tissues were subsequently transferred to cellosolve. Following fixation the specimens were carefully examined for tumours. Transverse blocks of the bowel were taken through each tumour, and through non-neoplastic bowel at two sites prone to tumour development, *viz.* 20 mm distal to the pylorus, and at the junction of the middle and lower thirds of the colon. Corresponding blocks were obtained from the control group. All these blocks were processed routinely to paraffin wax; histological sections prepared at 4 μm were stained with haematoxylin and eosin prior to counting.

Counting procedures and statistical analysis

The histological sections were counted as follows: Whole circumferential sections of non-neoplastic mucosa from either control or DMH-treated animals were counted for the low doses of vincristine, and half or one third circumferences for higher dose groups where metaphase arrest was more complete and mitotic figures thus much more numerous. Total numbers of mitotic figures in metaphase, arrested metaphase, anaphase and telophase were obtained, together with the total of unequivocal post-metaphase figures. The morphology of prophase is difficult to define, and it was decided to exclude this phase of mitosis from the study, although the possibility remains that some late prophase figures may have been included as metaphases. Using the counting methods described, the number of all mitoses counted in each section was 100–200 for low-dose groups and 200–300 for high dose groups. In the case of tumours, areas of viable neoplastic tissue were selected at hazard and similar counts made. The total mitoses counted for each tumour was 100–300. In the subsequent analysis the total mitoses are designated 'm' and total post-metaphase figures 'a'.

The ratio of post-metaphase figures (anaphases and telophases) to all mitoses is the *Post-Metaphase Index* (PMI $= a/m$). The relationship between the PMI, which is an index of the degree of escape from metaphase arrest, and the dose of vincristine was analysed using the computer program GLIM (Baker & Nelder, 1978). The PMI was transformed to the logit

$$\text{logit PMI} = \log_e\left(\frac{\text{PMI}}{1-\text{PMI}}\right)$$

$$= \log_e\left(\frac{a}{m-a}\right).$$

We selected the logit transformation, rather than a $\log_{10}$ transformation because of the nature of the observations. Thus for each observed mitotic figure there are two 'all or nothing' options *viz.* metaphase or post-metaphase. This yields data in the form of a biological assay with quantal responses for which one possible transformation is the logistic (Finney, 1978). We found that this transformation together with transformation of vincristine dose to $\log_{10}$ dose, was the most satisfactory in order to linearise our data. These transformed data were plotted and the slopes of the fitted lines calculated by the computer model. The data for small bowel and large bowel were analysed separately: at both sites there are three tissues represented, comprising normal mucosa, DMH-treated but not neoplastic mucosa, and tumour. These were designated Control, DMH, and Tumour respectively. In both small and large bowel analysis of deviance was performed to test for the significance of differences between the slopes of the fitted lines for each of the three types of tissue. Finally, the fitted lines were used to estimate the

doses of vincristine required to achieve a PMI of 1% (−logit PMI 0.01 = 4.60) for each tissue, and the result expressed as a ratio of control. A PMI of 1% is achieved in the middle of the range of the doses used.

Results

In the untreated animals there were no tumours. The number of tumours in the DMH-treated animals is shown in Table I. A total of 95 tumours was identified in 39 animals, 29 of the DMH-treated group being tumour free. The same range of tumour morphology was observed as that seen previously in DMH-treated rats (Sunter *et al.*, 1978), but all the variants were analysed together.

Table I Distribution of intestinal tumours in the DMH-treated animals by anatomical site and vincristine dosage group

Anatomical site	*No. of tumours*						
	Vincristine dose (mg kg^{-1} body weight)						
	1.00	*0.50*	*0.25*	*0.10*	*0.05*	*0.01*	
Colon	11	9	1	9	8	11	
Small intestine	18	2	5	7	5	9	
Total	29	11	6	16	13	20	(95)

The mean PMI for each combination of site and tissue at each dose of vincristine, together with the standard error, is shown in Table II. For each of the tissues there is a progressive rise in metaphase escape (an increasing PMI) as the dose of vincristine administered decreases. This relationship is not linear, there being a rapid increase in PMI over the lowest two doses. Over the whole range of doses tumour tissue from both anatomical sites shows more metaphase escape than either of the other two tissues. At high, and intermediate doses, the DMH-treated tissue also shows increased escape over control tissue, although the effect is not as marked. The PMI in each tissue in the absence of vincristine, i.e. the 'native' PMI, was not determined, except for a few DMH-treated animals killed prior to vincristine administration for humane reasons. The mean PMI of mucosa from these animals (corresponding to DMH in Table II) was 15% in the colon and 19% in the small intestine. These latter data have not been incorporated in the analysis; they were derived from animals killed before the full course of DMH-treatment, and no similar data are available for tumour or control tissue. Despite the imprecision and possible inaccuracy inherent in these estimates, it appears that the lowest vincristine dose may be associated with some measurable metaphase arrest in this tissue.

Because the relationship between PMI and dose

Table II Effect of vincristine dose on the PMI (%) in each site/tissue combination. Standard errors are included in parenthesis, and were calculated by the method of Snedecor and Cochran (1971)

Vincristine dose (mg kg^{-1})	*PMI %*					
	Colon			*Small intestine*		
	Control	*DMH*	*Tumour*	*Control*	*DMH*	*Tumour*
0.01	19.36 (0.92)	10.25 (0.93)	11.86 (0.91)	7.85 (1.05)	7.90 (0.92)	14.72 (0.80)
0.05	2.24 (0.48)	3.43 (0.41)	7.38 (0.90)	0.62 (0.15)	1.49 (0.20)	6.81 (1.03)
0.10	0.23 (0.10)	0.76 (0.18)	4.13 (0.63)	0.17 (0.09)	0.19 (0.07)	2.19 (0.55)
0.25	0.07 (0.06)	0.36 (0.10)	0.30 (0.15)	0.00	0.03 (0.02)	1.13 (0.26)
0.50	0.00	0.09 (0.04)	0.25 (0.12)	0.00	0.00	0.00
1.00	0.00	0.07 (0.05)	0.19[a] (0.06)	0.00	0.06 (0.04)	0.24[a] (0.11)

[a]A single animal in this group bearing 2 colonic and 4 small intestinal tumours showed a mean tumour PMI of 6.2%. Either vincristine administration was faulty, or these tumours were exceptionally resistant. We have excluded the animal from the analysis and from this table.

is non-linear two options for statistical analysis were considered *viz.* simple significance testing between mean PMI's at individual dosage points, or transformation of the data to achieve linearity with subsequent significance testing for differences between the slopes of the fitted lines. We have chosen the second option because it enables us to look for differences between tissues in their response to vincristine over the whole range of doses, using all the data for each test of significance.

Application of the GLIM program, using the transformation logit PMI and $\log_{10}$ dose, shows the relationship between the transformed values of PMI and vincristine dose to be linear. The fitted lines for each site/tissue combination are shown in Figure 1. Because the analysis gives more weight to data points for which larger numbers of post-metaphase figures were seen, i.e. in low dose groups, the fitted lines lie somewhat below the data points. We have plotted the negative logit PMI because the PMI is a measure of escape from metaphase arrest, and we prefer to consider the phenomenon in terms of increasing arrest plotted against increasing vincristine dose. At any given dose of vincristine tumour tissue shows the least degree of metaphase arrest, and control mucosa the most. DMH-treated, non-neoplastic mucosa shows an intermediate degree of vincristine sensitivity. For control and DMH-treated mucosae metaphase arrest appears to be rather more complete in the small intestine than in the colon for the same dose of vincristine. There is little difference in this respect between small-intestinal and colonic tumours. Analysis of deviance was used to test for differences between the slopes of the fitted lines at each anatomical site and the results are shown in Table III. Significant differ-

Table III Comparison of slopes of fitted dose response lines. The differences between slopes are shown, together with the standard errors of the differences

Site	*Control-DMH*	*Control-Tumour*	*DMH-Tumour*
Colon	1.44 ± 0.36^{a}	2.13 ± 0.34^{a}	0.69 ± 0.20^{a}
Small intestine	0.85 ± 0.46^{b}	1.97 ± 0.43^{a}	1.12 ± 0.22^{a}

[a]$P < 0.001$; [b]Not significant.

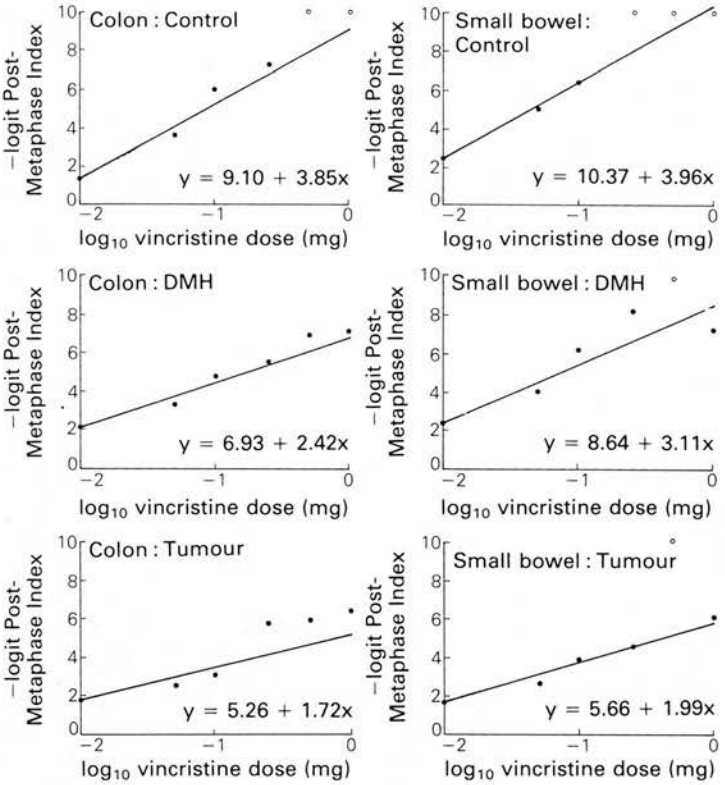

Figure 1 The transformed data points, fitted lines and slopes of the fitted lines are shown for each combination of site and tissue. Points marked o indicate a PMI of 0%, i.e. – logit PMI = ∞. All the points are included in calculating the fitted lines.

ences in sensitivity to vincristine are shown in the colon between all tissues, and in the small bowel between all but control and DMH-treated mucosae. The equations of the fitted lines were used to estimate the relative vincristine doses required, as a proportion of control tissue requirement, to achieve a PMI of 0.01. For tumour tissue the increased dose required is 6 times that of control mucosa in the colon, and 8 times in the small intestine. The corresponding values for DMH-treated mucosa are 1.6 times in the colon and 1.4 times in the small intestine.

Discussion

The PMI and tumour resistance to vincristine

We have shown, using a method measuring directly the escape of dividing cells from metaphase arrest, that induced primary tumours of the large and small bowel are more resistant to the stathmokinetic effect of vincristine than normal mucosa. Carcinogen-treated, but non-neoplastic mucosa shows an intermediate degree of vincristine resistance. These results are in keeping with other data derived from human colorectal tumour tissue and normal mucosa, but using a different method of assessing vincristine resistance (Pritchett *et al.*, 1982). That study showed an *in vitro* requirement for complete stathmokinesis of $3.0\,\mu g\,ml^{-1}$ for tumours and $0.5\,\mu g\,ml^{-1}$ for mucosa. Comparable data from the study of inherent vincristine resistance in murine colonic tumour cell lines shows an enhanced cytotoxic dose requirement of up to approximately tenfold in sensitive cell lines (Tsuruo *et al.*, 1983*a*).

From *in vitro* studies in cell lines biochemical and pharmacological data have convincingly demonstrated a cellular biochemical mechanism underlying vincristine resistance. The resistance of primary solid tumours to chemotherapy has been attributed, at least in part, to other factors. Poor tumour vascularity (Selby *et al.*, 1979) and changes in vascularity following therapy may be relevant, together with the cell-cycle phase dependency of some chemotherapeutic agents (Valeriote & Van Putten, 1975). Consideration of methodology, in both the present study and in our former study (Pritchett *et al.*, 1982) provides some insight into the possible contributions of these various factors towards the phenomenon that we have described. Some effect due to poor tumour vascularity is not excluded in the present study and we have not ascertained tissue concentrations of vincristine. Thus the data relates to administered doses which may not reflect tissue levels. Clearly this will not affect differences between DMH-treated and normal mucosae, and in the *in vitro* system it is irrelevant. Given the dependence of the experimental system on the perturbation of mitosis induced by vincristine, the cell-cycle phase-specificity of this mechanism is axiomatic. The reported observations relate to the effects of a first, and brief, exposure to vincristine. There can be no question, therefore, of induced alterations of tumour vascularity or cell-cycle synchronisation. We conclude that the phenomenon we have demonstrated reflects one aspect of vincristine resistance at an underlying cellular level.

The relationship between the cytotoxic and the stathmokinetic actions of vincristine is not resolved. The doses required to achieve complete metaphase arrest in various human and rodent epithelia are in considerable excess of those used as maximum doses in human cancer therapy. Despite this, such therapeutic doses are efficacious as cytotoxic agents to some solid tumours. Furthermore it has been shown that sensitivity to the Vinca alkaloids is manifest in G_1 and in S, as well as in the mitotic phase of the cell cycle (Mauro & Madoc-Jones, 1970). Vincristine is also associated with other non-cycle related metabolic disturbances within the cell which may be of importance in cytotoxicity. The pragmatic issue in terms of the potential usefulness of the PMI in the investigation of drug resistance is not whether stathmokinesis and cytotoxicity are equivalent, but rather whether resistance results from the same mechanism in both. Pharmacological manipulation of vincristine resistance using cell lines shows that a number of drugs including many calcium transport antagonists will overcome it, and cause increased intra-cellular accumulation of vincristine. We are presently investigating the effect of verapamil on the PMI of colonic tumour tissue using both human and animal models.

The validity of this approach to vincristine resistance using the PMI depends upon several factors: Does vincristine alter the flux of cells through the cell cycle? Is there any evidence that vincristine causes disproportionate prolongation of any of the morphological phases of mitosis other than the production of metaphase arrest? Are there any differences in the 'native' PMI of different tissues of sufficient magnitude to affect interpretation of the results? The extensive literature concerning the use of vincristine as a stathmokinetic agent has been comprehensively reviewed (Wright & Appleton, 1980). Although the specific problems generated by the present study have not been discussed previously, there is indirect evidence to help answer the above questions.

High doses of vincristine inhibit the flux of lymphoid cells through G_2 by inhibition of DNA

synthesis (Fitzgerald & Brehaut, 1970), and are also associated with increased degeneration of arrested metaphase figures (Smith *et al.*, 1974; Clarke, 1971). However, the doses used in the present experiment are in the range of the 'standard' doses described by Jellinghaus *et al.* (1977), and Clarke (1971), and do not affect the flux of cells into S-phase in the rodent intestine.

There is no evidence in the literature to suggest any effect of vincristine on mitotic phase duration, other than the primary effect on microtubule formation and inhibition of anaphase.

Pozharisski *et al.* (1982) while studying kinetic aspects of carcinogenesis in non-neoplastic mucosa using the DMH model noted that the proportion of anaphases and telophases prior to DMH treatment changed only slightly following treatment (from 17% to 20%). In the present study we were able to demonstrate a 'native' PMI in DMH-treated colonic mucosa of 15% and in DMH-treated small intestine 19%. Despite the imprecision of these latter observations, it is possible that there may or may not be minor differences between the PMI's of individual tissues. Use of the GLIM analysis to compare the rates of change of PMI with increasing vincristine dose, rather than significance testing for differences between PMI's at individual vincristine dosage points eliminates this problem in the comparison of different tissues. Pozharisski further states that up to 60% of mitotic figures in DMH-treated tissues are abnormal. Certainly it would be anticipated that more chromosomal bridges would occur in tumour tissue. These would tend to reduce the proportion of metaphases generating normal post-metaphase figures. We have found a greater proportion of post-metaphase figures at each dose in tumour tissue despite this diluting effect of mitotic abnormality.

We would like to acknowledge the technical assistance provided by Mrs K. Elliott.

The manuscript was word processed by Miss B. Kennedy. This work was supported by a grant from the North of England Cancer Research Campaign (NECC 418021).

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Br. J. Cancer (1988), **57**, 348–352

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Verapamil sensitises normal and neoplastic rodent intestinal tissues to the stathmokinetic effect of vincristine *in vivo*

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Summary A morphological method has been developed allowing measurement of the effect on intestinal epithelia of vincristine. In routinely prepared tissue sections the proportion of mitotic events progressing beyond metaphase is counted by microscopy. When estimated over a range of doses of vincristine this post-metaphase index (PMI) can be used to compare the sensitivity of differing intact tissues. Intestinal tumours were induced in rats by chemical carcinogenesis. Administration of vincristine in the presence or absence of verapamil was performed in these tumour-bearing animals. Sections were prepared from colonic and small-bowel tumours and from normal mucosa. The results show that verpamil increases the sensitivity of the tissues studied to vincristine. A dose dependent effect of verapamil on vincristine sensitisation was demonstrated in colonic tissues. These findings indicate a shared pharmacological property between the resistance of primary tumour tissue and the multidrug-resistance phenotype.

'Multidrug-resistance' (MDR) to a variety of naturally occurring agents used in cancer chemotherapy is a major area of research interest (Ling *et al.*, 1983, Tsuruo, 1983). It has been shown to contribute to resistance to the Vinca alkaloids, anthracyclines and podophyllotoxins. Vincristine is one of the few drugs with any role in the chemotherapy of colonic carcinoma. The problem of primary resistance to this drug has severely limited its usefulness and deserves investigation. We are currently interested in the possible similarity, in terms of the underlying biochemical processes responsible, between this primary resistance phenomenon and MDR. Studies using cell lines of this latter acquired form of resistance demonstrate that many lipophilic drugs are able to abolish or reduce it (Skovsgaard *et al.*, 1984). The postulated mechanisms include an interaction with the cell plasma membrane, the site of a putative drug-elimination pathway of increased activity in resistant cells, or an effect upon influx of the cytotoxic agent or upon its cytosolic binding. Among the many drugs studied which demonstrate such a modifying action verapamil has been most frequently used. It has been shown to reduce resistance to a wide variety of cytotoxic drugs in many tumour cell lines.

We have previously reported a method of quantifying resistance to vincristine in primary solid tumour tissues using a morphological method which generates the post-metaphase index (PMI) (Ince *et al.*, 1985). By applying this technique to human colonic carcinoma tissue grown in organ culture we have shown that verapamil increases the degree of metaphase arrest induced by vincristine (Ince *et al.*, 1986).

In the present communication we have proceeded to investigate the extent to which verapamil-enhanced sensitivity to vincristine can be demonstrated by this morphological method in intact experimental animals. We report here data from two experiments performed *in vivo* on intestinal tumours induced by chemical carcinogenesis in rats. The first is a study of the effect of high dose verapamil on the sensitivity of tumour and normal mucosa to vincristine, over a range of doses. The second is a verapamil dose-response experiment performed at a single dose of vincristine. The results confirm enhanced resistance to vincristine of tumour tissue over normal mucosa and indicate a sensitising effect of verapamil *in vivo*.

Materials and methods

Rats and DMH-treatment schedules

Eighty-eight male Wistar rats (Olac Ltd, Bicester) were used in the two experiments. They were maintained throughout the experiment with unrestricted access to food (Breeders diet no. 3, Special Diet Services Ltd, Witham) and tap water. They were housed in a 12 h light/dark cycle, and all injections were administered between 0900 h and 1100 h. All the animals received a long-term, low-dose schedule of 1,2-dimethylhydrazine (Aldrich Chemical Co. Ltd, Poole) exposure, comprising 24 subcutaneous injections at one week intervals, of a dose of 15 mg (base) kg^{-1} body weight. The animals were aged between 6 and 10 weeks at the start of the DMH treatment schedule and weighed between 150 g and 220 g. At the time of PMI experiments they were aged between 34 and 39 weeks and weighed between 350 g and 550 g. The animals were left for a period of between 3 and 5 weeks after the last DMH injection before subsequent experimentation. We adopt this procedure to allow complete recovery from any acute toxic effects of DMH.

High dose verapamil experiment (experiment 1)

Twenty-six verapamil-treated animals all received verapamil (Abbott Laboratories, Queensbourough) at a dose of 100 mg kg^{-1} body weight, administered by intraperitoneal injection in a vehicle comprising ethanol 40% v/v in normal saline. Thirty-four control (i.e. verapamil-untreated) animals received an intraperitoneal injection of the same volume of the vehicle only. Vincristine (Oncovin, Eli Lilly Ltd, Basingstoke) was administered at one of the following doses; 0.5, 0.25, 0.1, 0.05, 0.01 mg kg^{-1} body weight, by i.p. injection. The drugs were administered in the following sequence: verapamil was administered 2 h prior to vincristine, and the animals were killed 2 h after vincristine injection. Thus the animals received verapamil or vehicle alone for 4 h, and vincristine in combination for the last two hours. This experimental protocol generates 10 permutations of drug administration (5 vincristine doses given either with or without verapamil). The animals were assigned to one of these 10 groups by a method adopted to optimise the distribution of colonic tumours between the groups. The experiment was performed in small batches selected at random sequentially over a number of days, with dissection of the colons to maintain a running total of tumours in each group. This procedure allowed the allocation of animals on subsequent days to groups where fewer tumours were present. A target of 6 colonic tumours in each group was achieved in 8 of the 10 groups.

The animals were killed by cervical dislocation and autopsy was performed immediately. The small bowel was dissected free and fixed unopened for at least 10 h in Carnoy's fluid. The colon was dissected free with a margin

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Received 8 September 1987; and in revised form, 11 December 1987.

of anal skin, opened along its length, and pinned to a cork board prior to Carnoy fixation. The tissues were subsequently transferred to cellosolve. Following fixation the specimens were carefully examined for tumours. Transverse blocks of the bowel were taken through each tumour, and through non-neoplastic mucosa at two sites prone to tumour development, *viz.* 20 mm distal to the pylorus, and at the junction of the middle and distal thirds of the colon. All these blocks were processed routinely to paraffin wax; histological sections prepared at 4 μm were stained with haematoxylin and eosin prior to counting.

Verapamil dose response experiment (experiment 2)

Twenty-eight animals received verapamil at one of the following doses; 100, 50, 25, 10, and 5 mg kg^{-1} body weight. All the animals received vincristine at a single dose of 0.1 mg kg^{-1} body weight. Otherwise the experiment was performed exactly as above.

Counting procedures and statistical analysis

The proportion of mitotic figures showing escape from metaphase arrest in each section of tumour or normal mucosa was obtained using the method that we have previously described (Ince *et al.*, 1985). The histological sections were counted as follows: whole circumferential sections of non-neoplastic mucosa were counted for the low doses of vincristine, and half or one third circumferences for the higher dose groups where metaphase arrest was more complete and mitotic figures thus much more numerous. Total number of mitotic figures was obtained, together with the total of unequivocally normal post-metaphase figures. The morphology of prophase is difficult to define, and it was decided to exclude this phase of mitosis from the study, although late prophase figures may have been included in the overall total. Using the counting method described the number of all mitoses counted in each section was 100–200 for low dose groups and 200–300 for high dose groups. In the case of tumours, areas of viable neoplastic tissue were selected at hazard and similar counts made. The total mitoses counted for each tumour was 100–300. In the subsequent analysis the total mitoses are designated 'm' and total normal post-metaphase figures 'a'.

The ratio of post-metaphase figures to all mitoses is the Post-Metaphase Index (PMI = a/m). The relationship between the PMI, which is an index of the degree of escape from metaphase arrest, and the dose of vincristine was analysed using the computer program GLIM (Baker & Nelder, 1978). The PMI was transformed to the logit;

$$\text{logit PMI} = \log_e \frac{(1-\text{PMI})}{(\text{PMI})}$$

$$= \log_e \frac{(\text{m}-\text{a})}{(\text{a})}$$

We selected the logit transformation rather than a simple logarithmic transformation because of the nature of the observations. Thus for each observed mitotic figure there are two 'all or nothing' options *viz.* metaphase or post-metaphase. This yields data in the form of a biological assay with quantal responses for which one possible transformation is the logistic (Finney, 1978). We found that this transformation together with transformation of vincristine dose to $\log_{10}$ dose, was the most satisfactory in order to linearise our data. These transformed data were plotted and the slopes of the fitted lines calculated by the computer model using the method of maximum likelihood (McCullagh & Nelder, 1983). The data for small bowel and large bowel were analysed separately; at both sites there are two tissues represented comprising DMH-treated non-neoplastic mucosa, and tumour. These were designated Mucosa and Tumour respectively. Analysis of deviance was performed in experiment 1 to test for differences between the slopes and positions of the fitted lines. The *t*-test was used based on this analysis to test for significant differences between the tissues, the sites, and between verapamil-treated and untreated tissues.

Results

Experiment 1

The distribution of tumours and animals bearing tumours among the experimental groups is shown in Table I. Altogether 125 tumours were identified, 71 in the colon and 54 in the small bowel. The same range of tumour morphology was observed as that seen previously in DMH-treated rats (Sunter *et al.*, 1978), but all the variants were analysed together.

The mean PMI for each combination of state and tissue at each dose of vincristine, with or without verapamil, together with the standard error is shown in Table II. The data show a progressive rise in metaphase escape (an increasing PMI) as the dose of vincristine administered decreases. This corresponds with our previous experience of this kind of data (Ince *et al.*, 1985; 1986). In 15 of the 20 pairs of data (verapamil-treated *vs.* verapamil-untreated) there is less metaphase escape (i.e. a lower PMI) in the verapamil treated tissues. The dose-response relationship both in the presence and absence of verapamil is not linear. In order to test for a statistically significant difference between these dose response curves the data were transformed to linearity using the transformations of logit PMI and $\log_{10}$ dose as previously described. Figure 1 shows the transformed data points for one combination of tissue and treatments, viz. colon – tumours – verapamil, together with the fitted line calculated by the computer model. The fitted lines for all the transformed data are shown in Figures 2 and 3. We have plotted the negative logit PMI simply because we prefer to consider the phenomenon in terms of increasing metaphase arrest plotted against increasing vincristine dose.

Lines were fitted for the four highest doses of vincristine only. This is because of the minimal degree of metaphase arrest present at the lowest dose (see Figure 1). The fitted lines representing verapamil treated tissue lie parallel to the untreated. At the lowest dose of vincristine this relationship changes because the degree of escape from metaphase arrest is virtually maximal at this dose. Thus the PMI for 0.01 mg kg^{-1} vincristine is insufficiently different from the 'native' PMI for an enhancing effect to occur. The figures show that for both tumour tissue and non-neoplastic mucosa, in both sites, the fitted lines for the verapamil-treated tissues lie above their control counterparts. These differences are not marked and, when tested taking each pair individually, do not achieve statistical significance. However, the GLIM program was used to generate the following mathematical function which summarises the whole of the data:

$$\begin{aligned} -\text{logit PMI} = 11.50 &+ 0.39 \text{ if small bowel} \\ &- 5.36 \text{ if tumour} \\ &+ 0.36 \text{ if verapamil} \\ &+ (6.50 - 3.46 \text{ if tumour}) \times \log_{10} \text{ dose} \end{aligned}$$

In addition the program generates standard errors of these parameters allowing simple significance testing as follows:

parameter		s.e.	df	*t* value	
small bowel	0.39	0.15	26	$t = 2.53$	$P < 0.05$
tumour	−5.36	1.33	26	$t = 4.02$	$P < 0.001$
tumour (slope)	−3.46	1.08	26	$t = 3.20$	$P < 0.01$
verapamil	0.36	0.16	26	$t = 2.21$	$P < 0.05$

This analysis uses the whole of the data together and shows significant reduction in vincristine resistance in the

Table I Distribution of intestinal tumours and of tumour bearing animals in experiment 1 by anatomical site, and verapamil and vincristine dosage group

Anatomical site	*Verapamil*	*a. No. of animals* *b. No. of animals with tumours* *c. No. of tumours*																		
		Vincristine dose (mg kg^{-1} body weight)																		
		0.5			*0.25*			*0.1*			*0.05*			*0.1*			*(Total)*		
		a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Colon	treated	6	5	6	3	3	9	6	4	9	5	2	6	9	4	5	29	18	35
	untreated	4	4	8	6	4	7	8	6	8	7	5	10	9	3	3	34	22	36
Small intestine	treated	6	3	5	3	2	3	6	3	4	5	2	2	9	6	9	29	16	23
	untreated	4	2	3	6	4	6	8	5	8	7	4	7	9	5	7	34	20	31

Table II Effect of vincristine dose on the PMI% in the presence or absence of verapamil in each site/tissue combination. Standard errors are included in parenthesis and were calculated by the method of Snedecor and Cochran (1971)

	PMI%							
	Colon				*Small intestine*			
	tumour		*mucosa*		*tumour*		*mucosa*	
Vincristine dose (mg kg^{-1})	*treated*	*untreated*	*treated*	*untreated*	*treated*	*untreated*	*treated*	*untreated*
0.0	16.0 (1.2)		15.0 (1.7)		16.8 (2.5)		18.5 (2.1)	
0.1	11.5 (1.3)	15.5 (2.4)	9.5 (1.8)	11.3 (1.6)	14.2 (1.9)	13.8 (2.1)	8.1 (2.0)	7.8 (3.1)
0.05	8.2 (1.4)	10.5 (1.5)	4.4 (1.5)	4.5 (2.2)	3.8 (0.2)	6.5 (2.5)	3.3 (1.5)	2.3 (1.4)
0.10	2.9 (0.9)	3.1 (1.2)	0.3 (0.1)	0.9 (0.5)	1.1 (0.4)	5.1 (0.1)	0.2 (0.1)	0.4 (0.3
0.25	0.8 (0.3)	1.7 (0.9)	0.2 (0.1)	0	0.1 (0.1)	1.0 (0.5)	0	0.1 (0.1)
0.5	0.2 (0.1)	0.5 (0.2)	0	0	0.5 (0.4)	0.2 (0.1)	0	0

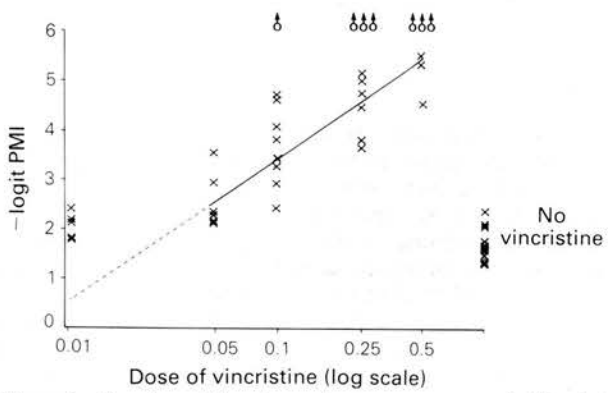

Figure 1 Experiment 1 colon – tumours – verapamil. The data points and fitted line for one combination of tissue and treatment. The circles and arrows represent PMI values of 0 (i.e. logit PMI $= -\infty$). The lowest dose of vincristine (0.01) shows PMI values approaching that of a group of verapamil only controls. This latter group has been transposed to the right hand side for clarity. The dotted section of the line represents an extrapolation from the line fitted between 0.05 and 0.5 ($\log_{10}$ mg kg^{-1} body wt.) at lower doses of VCR. It clearly does not correspond to the data over this dose range.

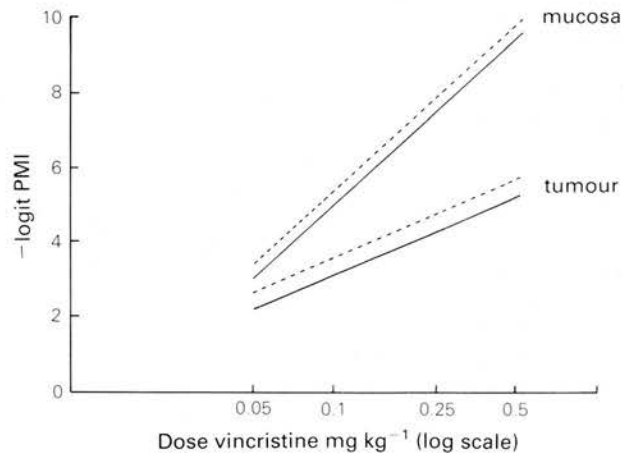

Figure 2 Experiment 1 – colon. All 4 fitted lines for verapamil treated (-----) and control (——) tissues. The higher the position of a line the greater the degree of metaphase arrest, i.e. the greater the sensitivity to vincristine.

presence of verapamil at an administered dose of 100 mg kg^{-1} ($P<0.05$). In addition the data confirm our previously reported findings of relative vincristine resistance in the colon compared with the small bowel ($P<0.05$), and in tumour tissue compared with normal mucosa ($P<0.001$).

Experiment 2

The distribution of intestinal tumours between the verapamil dose groups is shown in Table III. A total of 75 tumours were identified and a goal of 7 colonic tumours per dose group was achieved in 3 of the 5 groups. As previously described all morphological groups of tumours were analysed together.

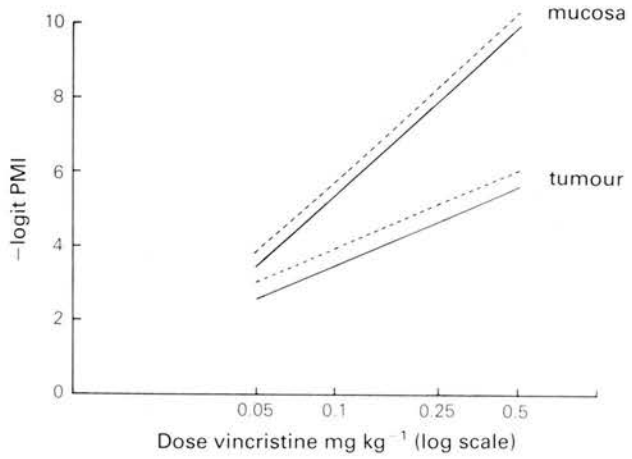

Figure 3 Experiment 1 – small intestine. As **Figure 2**, verapamil-treated (-----) and control (——) tissues.

The mean PMI% for tumours at each dose of verapamil is shown in Table IV. At this dose of vincristine (0.1 mg kg^{-1}) there is only minimal escape from metaphase arrest in non-neoplastic mucosa and this tissue was excluded from the analysis. The mean PMI% for the tumour tissues in this experiment at the verapamil dose given in the first experiment are 1.9 in the colon and 1.2 in the small bowel, and they are not significantly different from the values for the same vincristine dose (verapamil-treated) in experiment 1 of colon 2.9, and small bowel 1.1. We examined the data using the mathematical transformation as described above to look for a significant decrease in escape from metaphase arrest with increasing dose of verapamil. This is illustrated in Figures 4 and 5. The individual data points are included as well as the fitted lines. The fitted lines illustrated are curved because the abscissa plots the untransformed PMI%. The fitted line for colonic tumours shows the expected effect, that for small bowel tumours shows an apparent reversal of this trend. The equations describing these lines are as follows:

$$\text{colon} \quad \text{logit PMI} = -2.98 - 0.74 \times \log \text{ dose}$$

$$\text{small bowel} \quad \text{logit PMI} = -4.36 + 0.23 \times \log \text{ dose}$$

Using analysis of deviance standard errors for the slopes of the lines were calculated as follows:

parameter		s.e.	df	t value	
slope-colon	−0.74	−0.35	29	2.09	$P<0.05$
slope-small bowel	0.23	−0.33	28	0.71	NS

Our analysis shows a significant slope of the fitted line for the colonic tumours but not for small bowel tumours. Thus we have demonstrated a slight increase in the sensitivity to vincristine in colonic tumours in the presence of increasing doses of verapamil. The failure to demonstrate an effect in small bowel tumours in this experiment may be due to the rather small amount of data available but, in view of the negative value for the slope, it probably reflects the lower level of vincristine resistance present in this tissue.

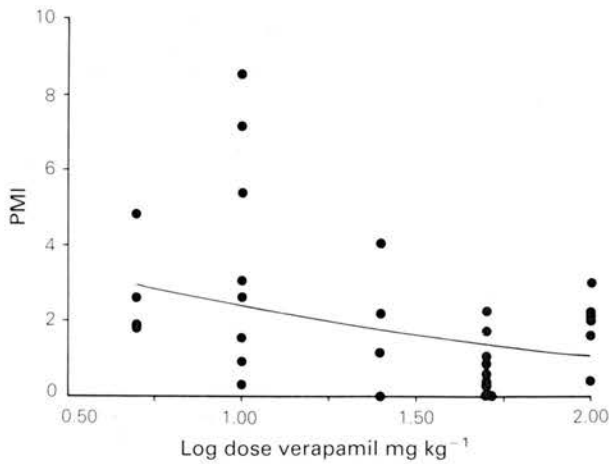

Figure 4 Experiment 2 – colon. Data points and fitted lines for tumour tissue. The ordinate shows the untransformed PMI thus the fitted line appears curved. The negative slope of the line is significantly different from 0 ($P<0.05$).

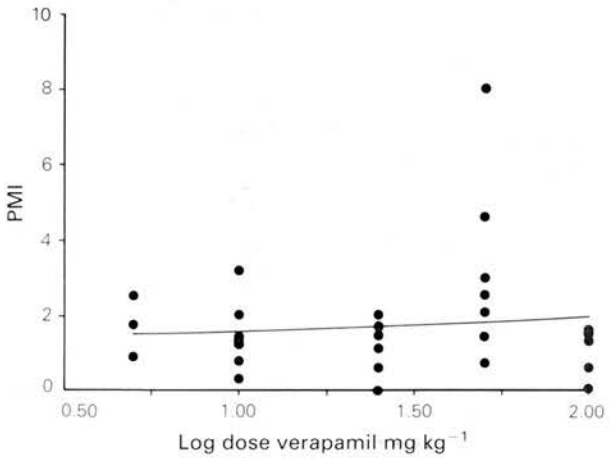

Figure 5 Experiment 2 – small intestine. Data points and fitted line for tumour tissue. The ordinate shows the untransformed PMI thus the fitted line appears curved. The positive slope is not significantly different from 0 ($P>0.05$).

Discussion

We have previously shown that the PMI offers a morphological means of measuring the degree of resistance to the stathmokinetic effect of vincristine within a tissue (Ince *et al.*, 1985). This method has the advantage over biochemical methods of estimating parameters related to drug resistance in intact tissues in that the measurements made relate exclusively to the epithelial tissue. Our previous work has also shown that the technique is sensitive enough *in vitro* to show differences between the resistance of tumour tissues when treated with a sensitising agent as compared

Table III Distribution of intestinal tumours and tumour bearing animals in experiment 2 by anatomical site and verapamil dosage group. All animals received vincristine at 0.1 mg kg^{-1}

Anatomical site	*a. No of animals* *b. No. of animals with tumours* *c. No. of tumours*																	
	Verapamil dose (mg kg^{-1} body weight)																	
	100			*50*			*25*			*10*			*5*			*(Total)*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Colon	5	3	10	6	3	10	5	4	5	6	3	9	6	3	4	28	16	38
Small intestine	5	2	10	6	4	8	5	3	9	6	4	7	6	3	6	28	16	29

Table IV Effect of verapamil on the PMI% in tumour tissue at each site at vincristine dose of 0.1 mg kg^{-1}. Standard errors are included in parenthesis and were calculated by the method of Snedecor and Cochran (1971)

	PMI%				
	Verapamil dose (mg kg^{-1})				
Anatomical site	*100*	*50*	*25*	*10*	*5*
Colon	1.9 (0.9)	0.8 (0.7)	2.5 (1.4)	2.3 (2.9)	3.4 (1.5)
Small intestine	1.2 (1.0)	3.0 (2.7)	1.4 (0.7)	1.4 (1.0)	1.8 (0.8)

with control untreated tissue (Ince *et al.*, 1986). *In vivo* we have shown differences between normal and neoplastic tissues and between different regional types of epithelial tissues in the gut.

The first experiment described here shows that using the PMI it is possible to show a sensitising effect of verapamil ($P<0.05$). It is apparent from the data that this effect is small. We have shown a similar degree of tumour cell resistance compared with normal mucosa to that which we have previously reported. Verapamil does not reduce the resistance of tumour tissue to that of normal mucosa. The pharmacokinetics of verapamil are of importance in the interpretation of the experiment. The administered dose of 100 mg kg^{-1} resulted in the death of 3 animals in the treated group ($\sim$LD10). This is a high dose and was selected to ensure the maximum tissue levels compatible with an adequate survival to the end of the experiment. In clinical therapy the highest achievable dose of verapamil is roughly equivalent to 5–10 mg kg^{-1}. In the second experiment reported here this dose range is associated with minimal enhancement of vincristine sensitivity. Thus it would seem that an effective clinical application of modifiers will require compounds active at lower doses.

The data presented here show no distinction between the effect of verapamil on normal compared with neoplastic tissues. This may reflect a similar sensitising effect of verapamil on both tissues, but this remains to be established. The standard errors of the means are large, particularly so in experiment 2, and larger numbers of tumours and animals would be needed to resolve this problem. We are currently investigating this aspect of the resistance of normal mucosa using our explant organ culture system. In this context the high level of mRNA for the p-glycoprotein reported in both tumourous and normal human colonic tissues is of interest (Fojo *et al.*, 1987). This finding can be interpreted as evidence of a naturally occurring cellular mechanism to protect the colon, amongst other tissues, from the effects of plant toxins. Taken further the theory can be used to account for both the enhanced resistance of MDR cells, and the resistance of spontaneous tumours on the basis of increased activity of this normal mechanism. Many tissues show low levels of p-glycoprotein mRNA including haemopoetic tissue. If modulators can only act on tissues possessing the p-glycoprotein then the bone marrow should be spared the effects of enhanced sensitivity. The PMI technique provides an opportunity to investigate this therapeutically important point.

In terms of the investigation of the relationship between the innate resistance of primary solid tumours and the acquired MDR phenotype we have shown both here *in vivo*, and previously *in vitro* using human tissue (Ince *et al.*, 1986), that the innate resistance to vincristine of colonic cancer shares a pharmacological property with MDR, *viz.* modulation of resistance by verapamil. Other evidence is required to elucidate this link. Studies using monoclonal antisera to the MDR-related p-glycoprotein in patients relapsing from acute nonlymphoblastic leukaemia have demonstrated resistant tumour cells containing large amounts of antigen (Kartner *et al.*, 1985, Ma *et al.*, 1987). Blot analysis of a wide range of human tissues, both normal and malignant, has shown higher levels of cytoplasmic m-RNA coding for p-glycoprotein in adrenal, renal and colonic tissues compared with others (Fojo *et al.*, 1987). Biochemical studies have shown decreased drug accumulation in human tumour cells lines derived from haematological malignancies (Ferguson & Cass, 1985) and carcinoma tissue (Fojo *et al.*, 1985). However, there is no evidence concerning the biochemical activity of the p-glycoprotein in native colonic tumour cells or in normal colonic mucosa. We are currently investigating the accumulation, localisation and efflux of tritiated alkaloids in normal colonic mucosa to compare these properties with those described in MDR.

Histological sections were prepared by Mrs K. Elliot. Graphs were prepared by Mr W. Robinson, and secretarial assistance provided by Miss E. Wark. This study was supported by a grant from the North of England Cancer Research Campaign.

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David Appleton

JOURNAL OF PATHOLOGY, VOL. 156: 000–000 (1988)

45

A PROTECTIVE EFFECT OF SULINDAC AGAINST CHEMICALLY-INDUCED PRIMARY COLONIC TUMOURS IN MICE

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Received
Accepted

SUMMARY

Sulindac, a non-steroidal anti-inflammatory drug, has been reported to lead to tumour regression in cases of human polyposis coli. We have investigated the effects of this drug on the growth of 1,2-dimethylhydrazine (DMH)-induced mouse colonic tumours. In one experiment, DMH and oral sulindac were administered concurrently to a group of mice for a period of up to 24 weeks, while a control group of animals received DMH only for the same period. Sulindac caused a significant reduction in both the number of mice with colonic tumours and the number of tumours per mouse. In a second experiment, two groups of mice which had already been treated with DMH for 17 weeks received either sulindac or not for 78 days. In this experiment sulindac had no effect. These results demonstrate that sulindac has a protective effect against the chemical induction of colonic tumours in mice, but does not cause the regression of established tumours.

KEY WORDS—Polyposis coli, sulindac, dimethylhydrazine, non-steroidal anti-inflammatory drug, colonic tumours in mice.

INTRODUCTION

In 1983, Waddell *et al.*[1] reported the regression of large bowel polyps in four patients with Gardner's syndrome treated with sulindac, a non-steroidal anti-inflammatory drug (NSAID). The rationale of this treatment lay in the serendipitous finding that indomethacin, another NSAID, had caused the regression of a desmoid tumour in a patient with pericarditis.[2] Three patients underwent a subtotal colectomy and ileoproctostomy, and in two of these there was complete disappearance of three and eight rectal polyps respectively after treatment of up to 2 years. In one patient with more than 50 polyps, only three were present after 4 months' treatment. The fourth patient had diffuse adenomatosis of the intact colon and rectum; after a year's treatment, only three small polyps remained. A subsequent report in 1985[3] by Gonzaga *et al.* noted the disappearance of 'thousands of colorectal adenomatous polyps' in a case of familial polyposis coli, after treatment with sulindac for a year.

In earlier studies, several groups had investigated the effects of non-steroidal anti-inflammatory drugs on tumour growth using experimental models. Indomethacin and aspirin were shown to reduce the growth of transplanted P815 ascites tumour and Lewis lung carcinoma cell lines in mice;[4] they also inhibit cell proliferation in hepatocellular carcinoma and human fibroblast cell cultures.[5]

Pollard and Luckert[6] have reported a reduction in size and number of intestinal tumours in rats which had received five oral doses of 1,2-dimethylhydrazine (DMH) followed by 15 weeks of indomethacin as compared with a control group. Caignard *et al.*,[7] however, failed to demonstrate such an effect in a similar experiment.

DMH and its metabolites azoxymethane and methylazoxymethanol are well-documented carcinogens[8–10] which are commonly used to induce colonic tumours in rodents. In mice, they produce numerous adenomatous polyps in the distal half of

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0022-3417/88/000000-00$05.00
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the large bowel. In rats, they produce tumours in a variety of organs depending on the dosage and route of administration; when administered by subcutaneous injection;[11] they produce both large and small bowel tumours, but compared with the lesions in the mouse model these tumours are less numerous though proportionately more are malignant. Consequently, in this study we chose mice as the appropriate experimental animals in which to induce colonic tumours by parenteral administration of DMH, in order to simulate more closely the human condition of adenomatosis coli. In one cohort of mice, sulindac was administered concurrently with DMH to assess its effect on tumour development. In a separate cohort, sulindac was administered subsequently, after an initial period of DMH treatment, in order to assess any possible regression of established tumours.

MATERIALS AND METHODS

Animals

Female Balb C mice aged between 6 and 8 weeks from an established breeding colony were used in these experiments. They were housed in plastic containers and allowed unrestricted access to a standard diet (Special Diet Services RMIE 1, Stepfield Witham, Essex) and water. The average water intake was monitored for a week prior to the start of the experiment and estimated to be approximately 3 ml/animal/day.

DMH injections and treatment

1,2-Dimethylhydrazine hydrochloride (Aldrich Chemical Co. Ltd., Gillingham, Dorset) was dissolved in EDTA (0·4 per cent) and normal saline (0·9 per cent) and brought to pH 6·5 with sodium hydroxide. This solution was injected subcutaneously (25 mg DMH base/kg body weight) once weekly.

Sulindac, *cis*-5-fluoro-2-methyl-1-[*p*-(methylsulphinyl)-benzylidenyl]-indene-3-acetic acid (Merck Sharp and Dohme Ltd.), was dissolved in tap water in a concentration such that each animal received an average dose corresponding to 5 mg/kg a day.

In the first experiment, two groups of 48 animals were treated with DMH as described above, either with or without sulindac, for up to 24 weeks. In the second experiment, 48 animals were treated with DMH for 17 weeks; 45 of these survived. After a 10-day interval, they were randomized, taking body weight into account, and 22 were given sulindac solution for up to 78 days while 23 animals received water.

All animals were killed by neck dislocation at the end of the experimental period. If any animal showed evidence of distress prior to the completion of the experiment it was killed together with an animal of comparable weight from the other group in order to avoid bias. The intestinal tract was resected; the large bowel was opened longitudinally, pinned out on cork, and placed in Carnoy's fixative for at least 4 h before being transferred to cellosolve. After fixation, the colon was carefully examined and the number of macroscopically obvious tumours was counted. Each tumour was then measured in two dimensions with the aid of a dissecting microscope. Transverse blocks of the colon were taken to include each tumour. Where there was no tumour, a transverse block was taken from the mid-point of the distal quarter of the large intestine. These blocks were processed routinely to paraffin wax, sectioned at 4 μm and stained with haematoxylin and eosin.

Statistics

The results relating to tumour burden per mouse and the distribution of tumours were analysed using the Mann-Whitney *U* test. In experiment 1, the odds ratio (approximate relative risk) was calculated when comparing the numbers of mice with tumours in the two groups. Data matched within animals were analysed by the Wilcoxon signed rank test. Correlations were investigated using the Spearman rank correlation coefficient. We selected these tests because of the skewed distribution of the data.

For each colonic tumour the maximum longitudinal and transverse dimensions (d_1 and d_2) were obtained. In order to use these measurements in the estimation of tumour size, the following procedure was adopted. Assuming that each tumour can be represented as a hemisphere with a mean diameter $(d_1+d_2)/2$, its volume was determined as $(\pi/96)(d_1+d_2)^3$. For each tumour-bearing mouse, the tumour volumes were added and the diameter (d) of the hemisphere corresponding to that total volume was calculated, viz., $d=(1/2)\ 3\sqrt{\sum(d_1+d_2)^3}$. This value was used as a measure of tumour burden per mouse.

RESULTS

Experiment 1. Concurrent administration of DMH and sulindac

Number of animals with tumours (Table I)—There were 48 animals in each group at the start of

the experiment. Ten pairs of animals died or were killed prior to 23 weeks. Colonic tumours were present in only two of these animals killed at 22 weeks, both from the sulindac-treated group; they bore one and two tumours, respectively. The ten pairs included equal numbers of distressed animals from each of the two groups. Thus, 38 animals in each group survived to the end of the experiment. Among these, there were fewer animals with tumours in the sulindac-treated (42 per cent) than in the control (89 per cent) group (odds ratio = 11·7, 95 per cent confidence limits 3·5–39·6; and 10·4, 95 per cent confidence limits 4·2–34·2 if the two animals from the sulindac-treated group which died at 22 weeks are included).

Table I—Experiment 1 (concurrent DMH/sulindac): number of mice with tumours

	Control group	Sulindac group
Number of animals with tumours	34	16
Number of animals with no tumours	4	22
Total number of animals	38	38
Percentage with tumours	89	42

Odds ratio = 11·7, 95 per cent confidence limits 3·5–39·6; and 10·4, 95 per cent confidence limits 4·2–34·2 if the two animals from the sulindac-treated group which died at 22 weeks are included.

Number of tumours per animal (Table II)—There were fewer tumours per mouse in the sulindac-treated group than in the untreated group ($P < 0{\cdot}0001$). Even when only those animals bearing tumours were considered, there were fewer tumours per mouse in the treated than in the untreated group ($P \simeq 0{\cdot}022$).

Tumour burden per animal—The overall tumour burden per mouse (Table III) was reduced in the sulindac-treated group ($P < 0{\cdot}0001$), and when only those mice which developed tumours were considered the tumour burden per animal was again lower in the treated than in the untreated group ($P \simeq 0{\cdot}058$). This finding reflects a reduction in the number of tumours per mouse rather than a population of smaller tumours, as the median diameter of tumour per mouse (Table IV) was not significantly reduced ($P \simeq 0{\cdot}73$).

Table II—Experiment 1 (concurrent DMH sulindac): number of tumours per animal

No. of tumours	Control group	Sulindac group
0	4	22
1–4	16	13
5–8	10	3
9–12	6	—
13–16	1	—
17–20	1	—
Total	38	38

Mann–Whitney $U = 271$, $P < 0{\cdot}0001$. When only tumour-bearing animals are considered: Mann–Whitney $U = 162$, $P \simeq 0{\cdot}022$.

Table III—Experiment 1 (concurrent DMH/sulindac): tumour burden per animal

d	Control group	Sulindac group
0 (no tumour)	4	22
$0 < d \leqslant 1$ mm	0	2
$1 < d \leqslant 2$ mm	9	3
$2 < d \leqslant 3$ mm	8	7
$3 < d \leqslant 4$ mm	7	3
$4 < d \leqslant 5$ mm	4	1
$5 < d \leqslant 6$ mm	3	—
$6 < d \leqslant 7$ mm	3	—

Tumour burden was represented by the diameter d of the hemisphere whose volume was that of the sum of tumour volumes estimated in each animal (see text). Mann–Whitney $U = 289$ $P < 0{\cdot}0001$. When only those mice that developed tumours were considered: Mann–Whitney $U = 181$, $p \simeq 0{\cdot}058$.

Tumour sites—The tumours were situated exclusively in the distal half of the large bowel, predominantly within the distal one-quarter. There was no significant difference in the pattern of distribution between the test and the control group.

Histology—All 236 macroscopic tumours were shown to be benign adenomata except for ten

Table IV—Experiment 1 (concurrent DMH sulindac): median tumour diameter

Median tumour diameter (mm)	Control group	Sulindac group
<1	2	1
1·01–1·5	9	6
1·51–2·0	14	5
2·01–2·5	6	1
2·51–3·0	2	3
3·01–3·5	0	—
>3·5	1	—
Total	34	16

Mann Whitney $U = 288{\cdot}5$, $P \simeq 0{\cdot}73$.

adenocarcinomata with early invasion through the muscularis mucosae; seven of these were from the control group and the other three were from the sulindac-treated group. The adenomata showed a predominantly well-differentiated tubular pattern with occasional villous areas. A mild inflammatory infiltration of lymphocytes and histiocytes was present in the stroma, but did not form a distinct peripheral reaction. In some tumours, polymorphs were also present in the stroma together with a mild cryptitis and occasional crypt abscesses. Widespread dysplasia was noted in the intervening mucosa and there were also numerous microadenomata which consisted of irregular branched glands showing architectural dysplasia and cellular atypia.

Dysplasia and inflammation—No attempt was made to quantify accurately the degree of dysplasia and extent of inflammation; subjectively, however, there was no substantive histopathological difference as between the groups.

Microadenomata—Each histological section was carefully examined for microadenomata. There were fewer mice with microadenomata in the sulindac-treated group (47·4 per cent) than in the control group (92·1 per cent) (Table V) (odds ratio = 13·0, 95 per cent confidence limits 3·4–49·5). Furthermore, the median number of microadenomata per section was reduced in the sulindac-treated group (Mann–Whitney $U = 1221{\cdot}5$, $P < 0{\cdot}0001$). In both the test and the control groups, there was a significant positive correlation between the median number of macroscopic tumours per animal and the median number of microadenomata per section (Spearman rank correlation coefficient = 0·624, $P < 0{\cdot}0001$ in the test group; and 0·577, $P < 0{\cdot}004$ in the control group).

Table V—Experiment 1 (concurrent DMH sulindac): number of mice bearing microadenomata

	Control group	Sulindac group
Microadenomata present	35	18
Microadenomata not present	3	20
Total	38	38
Percentage with microadenomata	92·1	47·4

Odds ratio = 13·0, 95 per cent confidence limits 3·5–49·5.

The colons of tumour-bearing animals which had received sulindac were subjected to further histological examination. One extra transverse block was taken from the distal quarter of the large bowel such that a macroscopic tumour was not included; each histological section was then examined carefully for microadenomata.

The number of microadenomata in each of these non-tumour-bearing blocks did not differ significantly from the median number of microadenomata in tumour-bearing blocks from the same animal (Wilcoxon matched pairs signed-rank test $T = 223{\cdot}5$, $P = 0{\cdot}70$). This indicates that the distribution of microadenomata was not influenced by proximity to macroscopic tumours. In the test group, the numbers of microadenomata in these extra blocks were also compared with those in blocks from non-tumour-bearing animals, and were found to be significantly more numerous (Mann–Whitney $U = 85$, $P = 0{\cdot}0040$).

Experiment 2. DMH followed by sulindac

Two groups of 22 and 23 animals each, which had been treated with DMH for 17 weeks, received sulindac solution or water, respectively, for up to 78 days. If any became distressed, they were killed in pairs as previously described except for one animal in the control group. Those killed within 8 weeks of treatment were excluded from the experiment.

Thus, the results below relate to 5 pairs of animals killed at 11 weeks, 12 pairs at 9 weeks, and 1 pair at 8 weeks.

Number of mice with tumours (Table VI)—There was no significant difference between the control and sulindac-treated groups in the numbers of mice with tumours; only one mouse (in the control group) did not have any tumours.

Table VI—Experiment 2 (DMH then sulindac): number of mice with tumours

	Control group	Sulindac group
Tumours	17	18
No tumours	1	0
Total	18	18

Number of tumours per animal (Table VII)—There was no significant difference between the control and treated groups in the number of tumours per mouse ($P \simeq 0{\cdot}84$).

Table VII—Experiment 2 (DMH then sulindac): number of tumours per mouse

No. of tumours	Control group	Sulindac group
0	1	0
1–4	3	3
5–8	3	6
9–12	3	2
13–16	4	3
17–20	3	3
21	1	1
Total	18	18

Mann-Whitney $U = 155{\cdot}5$, $P \simeq 0{\cdot}84$.

Tumour burden per animal (Table VIII)—There was no significant difference in tumour burden between the sulindac and control groups ($P \simeq 0{\cdot}57$).

Table VIII—Experiment 2 (DMH then sulindac): tumour burden per mouse

d	Control group	Sulindac group
0 (i.e., no tumour)	1	0
$0 < d \leqslant 1$	1	0
$1 < d \leqslant 2$	0	1
$2 < d \leqslant 3$	4	2
$3 < d \leqslant 4$	1	5
$4 < d \leqslant 5$	3	1
$5 < d \leqslant 6$	3	5
$6 < d \leqslant 7$	3	2
$7 < d \leqslant 8$	1	2
$8 < d \leqslant 9$	1	—

The tumour burden per mouse is estimated as d (see Table III). Mann-Whitney $U = 144$, $P \simeq 0{\cdot}57$.

Tumour sites—In both groups of animals, the pattern of tumour distribution was similar to that described in experiment 1.

Histology—All 379 macroscopic tumours were shown to be benign adenomata except for six adenocarcinomas—five from the experimental group and one from the control group. Histological appearances in relation to dysplasia and inflammation were similar to those in experiment 1.

Number of microadenamota—There was no significant difference between the control (83·5 per cent) and sulindac-treated (72·2 per cent) (Table IX) groups in the numbers of mice with microadenomata (odds ratio = 1·9, 95 per cent confidence limits 0·4–9·6). Also there was no significant difference in the median number of microadenomata per section between the control and experimental groups (Mann-Whitney $U = 182{\cdot}5$, $P \simeq 0{\cdot}51$). In both groups, the correlation between the median number of macroscopic tumours per animal and the median number of microadenomata per section was positive, but not significant (Spearman rank correlation coefficients = 0·220. $P \simeq 0{\cdot}36$ and 0·35, $P \simeq 0{\cdot}15$ for the experimental and control groups, respectively).

DISCUSSION

In the cases reported by Waddell *et al.*[1] and Gonzaga *et al.*,[3] there was tumour regression in five patients with polyposis coli treated with sulindac.

Table IX—Experiment 2 (DMH then sulindac): number of mice with microadenomata

	Control group	Sulindac group
Microadenomata present	15	13
Microadenomata not present	3	5
Total	18	18
Percentage with microadenomata	83·3	72·2

Odds ratio = 1·9, 95 per cent confidence limits 0·4–9·6.

Three of these patients had previously undergone an ileoproctostomy, an operation which has been associated with tumour regression in the rectal stump,[12] but in the other two cases tumour regression would appear to be solely attributable to sulindac treatment. Although our experimental findings have failed to demonstrate this phenomenon, they do, however, demonstrate that sulindac has an inhibitory effect on the development of DMH-induced colonic tumours in mice. Both the number of mice with tumours and the number of macroscopic tumours and microadenomata were reduced when DMH and sulindac were administered together.

Following weekly injections of DMH, a number of changes take place within the mouse colonic mucosa.[10] There is an initial hyperplastic response with elongation of crypts. This progresses to dysplasia with goblet cell depletion, cellular and nuclear pleomorphism, nuclear hyperchromatism, loss of nuclear polarity, stratification of nuclei, and mild cytoplasmic basophilia. Further progression is followed by the multifocal formation of microadenomata. Each microadenoma consists of several atypical glands situated initially near the surface and showing marked cytoplasmic basophilia, nuclear stratification, and loss of nuclear polarity. These lesions are regarded as immediate precursors of macroscopically evident sessile adenomata. Some adenomata show a propensity for downward invasive growth through the muscularis mucosae and develop into adenocarcinomata.

In our study, impaired development of macroscopic tumours was paralleled by a reduction in the incidence and numbers of microadenomata. This would suggest that sulindac exerts its effect at that stage during tumorigenesis where there is progression from dysplasia to microadenoma formation; microadenomata arising in the presence of sulindac then progress to adenomatous polyps at the same rate as in the control group. The rate of growth of macroscopic adenomatous polyps is evidently unaffected by sulindac since there was no difference in the median tumour size between the two groups.

If this mode of action applies in the human colon, then there is an apparent discrepancy between the results of our experiments and the clinical case reports alredy referred to, in respect of the regression of established adenomata. This could be explained in terms of a hypothesis which postulates that in early adenomatosis coli, some of the adenomata are in a labile state and become more stable as the disease progresses. Thus, adenomata would regress spontaneously alongside newly forming lesions. It is the development of these new polyps which would be impaired by sulindac treatment, giving rise to a picture of apparent overall tumour regression. Our findings in experiment 2, in which sulindac failed to cause tumour regression, may be consonant with this postulated later stable phase in the life history of adenomata. Alternatively, there may be a substantive difference between human and murine adenomata in respect of their cytokinetic response to sulindac.

The exact biochemical action of sulindac in these experiments is not certain but it is possible that it is acting via inhibition of prostaglandin synthesis. Such an action may explain the beneficial response obtained when indomethacin is administered to mice with transplantable NC carcinoma cell lines.[13] Tumour cells are thought to escape host immune surveillance through the production of prostaglandins which are immunosuppressive.[14] It is therefore conceivable that sulindac, by reducing prostaglandin synthesis in colonic tumours, which contain more prostaglandins than adjacent mucosa,[15] restores normal immunological mechanisms in the host. There was no subjective evidence for this mechanism in this study as represented by an alteration in the nature of the cellular inflammatory infiltration of the mucosa and tumour stroma in the sulindac-treated animals. These changes are difficult to quantify; we do not exclude more subtle changes, for example involving T-lymphocyte subsets.

Further experiments are now indicated to investigate this phenomenon in terms of both the

biochemical mechanisms involved and the changes in proliferative parameters in colonic tissues exposed to sulindac.

ACKNOWLEDGEMENTS

This work was supported by a grant from the North of England Cancer Research Campaign. We would like to thank Mrs K. Elliott for technical work and Miss E. Wark for typing the manuscript. We are also greatly indebted to Merck Sharp and Dohme Ltd. for the supply of sulindac.

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David Appleton

The cell cycle time in the flat (avillous) mucosa of the human small intestine

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Gut, 1973, **14**, 603-606

The cell cycle time in the flat (avillous) mucosa of the human small intestine

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SUMMARY A hyperproductive mucosal state in gluten-sensitive enteropathy has been proposed on the basis of an elevated mitotic index, but this parameter is dependent on the mitotic duration when used as an index of proliferative status. The mitotic duration was therefore measured in two control patients with normal villous mucosae and in two patients with the flat avillous mucosa of untreated gluten-sensitive enteropathy, using two different stathmokinetic techniques with vincristine. No significant difference in mitotic duration was found but values obtained for cell cycle time showed a halving in the flat mucosae. An increased rate of cell production in the small bowel mucosa of untreated gluten-sensitive enteropathy is thus confirmed.

In a further article (Wright, Watson, Morley, Appleton, and Marks, 1973) we report an increased incidence of dividing crypt epithelial cells in the flat avillous mucosa of untreated gluten-sensitive enteropathy, so confirming earlier reports (Padykula, Strauss, Ladman, and Gardner, 1961; Yardley, Bayless, Norton, and Hendrix, 1962). On the basis of such findings the crypt cells in gluten-sensitive enteropathy have usually been supposed to have a shortened intermitotic or cell cycle time. But Winawer and Lipkin (1971) have recently cautioned that an increased mitotic incidence in gluten-sensitive enteropathy connotes a decreased cell cycle time only if the duration of mitosis remains constant. Under steady state conditions, the mitotic index (I_m) is related to the mitotic duration (t_m) and the cell cycle time (T_c), thus

$$I_m = \frac{t_m}{T_c} \qquad \ldots(1)$$

An increase in mitotic index could therefore be caused by an increase in mitotic duration as well as by a reduction in cell cycle time. It is clearly necessary to make direct measurements of t_m and T_c. In this paper we report measurements of these parameters in two patients with morphologically normal small bowel mucosa, and in two patients with the flat mucosa of untreated gluten-sensitive enteropathy.

Received for publication 3 May 1973.

Materials and Methods

The full experimental procedure was explained in detail to the four subjects before informed consent was obtained. Two procedures, both employing the metaphase-arresting properties of vincristine (Oncovin, Lilly), were used to measure T_c and t_m in these four patients.

Patient A was a male aged 61, with a clinical diagnosis of rosacea and leg ulceration resulting from stasis.

Patient B was a male aged 37 who had suffered from dermatitis herpetiformis for over 10 years, and who had recently presented with a megaloblastic anaemia due to folic acid deficiency. He had been treated with Dapsone and had been eating a normal diet.

On day 1 at 13.50 hr a peroral biopsy was taken from the duodenojejunal region of each patient using a modified Crosby capsule. On day 2 the capsule was again passed to the same position under radiological monitoring. Then at 11.00 hr vincristine sulphate was administered by rapid intravenous infusion in a dose of 0·045 mg per kg body weight. At 13.50 hr the capsule was fired and a mucosal biopsy obtained.

Patient C was a male aged 61, who was being investigated for diarrhoea.

Patient D was a female aged 55 with a clinical diagnosis of untreated coeliac disease.

In these two patients a different procedure was used. The capsule of a Quinton hydraulic multiple suction biopsy machine was passed into the jejunum and a base-line biopsy obtained at 1100 hours. Vincristine sulphate was immediately given in the same dose as before by rapid intravenous injection. Serial mucosal biopsies were then obtained at approximately 15-min intervals.

All biopsies were orientated under the stereomicroscope and spread on glass, fixed in 10% neutral buffered formalin, and postfixed in Carnoy's solution. The tissue was wax embedded, and serial sections were cut at a thickness of 3 μm and stained with Harris's haematoxylin. The counting techniques used for analysis of the biopsies from patients A and B are described in a further paper (Wright *et al*, 1973). In patients C and D, at least 3000 crypt nuclei were counted in each biopsy. Only optimally sectioned crypts, ie, those with the base, middle, and mouth present in the plane of section, were analysed.

Results and Interpretation

In patient A both biopsies showed only finger- and narrow leaf-shaped villi, while histological examination showed no notable abnormality. Patient B had a 'flat' mucosa with a cobblestone appearance in both samples and the histological appearances were characteristic of untreated gluten-sensitive enteropathy.

The cytokinetic data are summarized in the table. The native or resting mitotic index ($I_{m(o)}$), obtained on day 1 in each case, was 2·36% in patient A and 5·09% in patient B ($P < 0.001$). In these biopsies all phases of mitosis were counted. In the biopsies after vincristine taken after 2·5 hr in each case, these values were raised to 7·75% and 14·81% respectively. In these samples no anaphases or telophases were seen and metaphase arrest was concluded to be complete. Only prophases and metaphases were therefore counted. Degeneration of arrested metaphases was not encountered (Aherne and Camplejohn, 1972), probably as a consequence of the short arrest period.

The mitotic duration t_m can be calculated from the following expression (Lala, 1971), assuming steady state conditions.

$$t_m = \frac{I_{m(o)} \cdot t}{(I_{m(t)} - I_{m(o)})} \quad \ldots(2)$$

where t is the period of metaphase or stathmokinetic arrest, in each case 2·5 hours. From this equation the mitotic duration in patient A was 1·09 hr and in patient B, 1·31 hr ($P > 0.05$). It cannot be stated therefore that there is a real increase in t_m.

The cell cycle time can now be calculated from equation 1. In this calculation it is necessary to incorporate Tannock's constant (Tannock, 1967), a factor by which I_m must be multiplied to compensate for the spatial distribution of mitoses and interphase small intestinal mucosal cells; this is because mitotic cells move towards the crypt axis, and would therefore be counted preferentially in sections of the crypt. Measured by the method of Tannock (1967) the constant was 0·71 in patient A and 0·72 in patient B.

Now the apparent cell cycle time ($T_{c(a)}$) is the cell cycle time if all the crypt cells are proliferating, ie, if the growth fraction or proliferating population is equal to 1. $T_{c(a)}$ was accordingly found to be 64 hr in patient A and 36 hr in patient B ($P < 0.001$). The growth fraction can be approximately determined from mitotic index distribution curves (Wright *et al*, 1973) and was found to be 0·83 in patient A and 0·61 in patient B. As noted elsewhere (Wright *et al*, 1973) these are maximum values for the growth fraction. Such estimates of the proliferating population can be used to modify the mitotic index, so that it deals only with the crypt cells actually dividing, by substituting in the expression

$$I_m = \frac{N_m}{(N_p/N_t)N_t} \quad \ldots(3)$$

where I_m is the corrected mitotic index, N_m the number of cells in mitosis, N_p the number of proliferating cells, and N_t the total number of cells (Gavasto and Pileri, 1971). The cell cycle times then become 54 hr in patient A and 22 hr in patient B.

The use of equation 2 to calculate t_m assumes not only that vincristine exerts its stathmokinetic effect immediately upon injection, but also that linear accumulation of metaphases occurs thereafter. These assumptions were investigated in patients C and D using the multiple biopsy technique.

In patient C 11 serial biopsies were obtained over a period of 150 minutes. Stereomicroscopy showed in all specimens a predominance of finger- and narrow leaf-shaped villi, with a small minority of broader leaves. Histological examination showed no abnormality. In patient D nine serial biopsies were obtained over a period of 105 minutes. All specimens showed a flat mucosa on stereomicroscopy, and the appear-

	Total Cells Counted	*Cells in Mitosis*	*Mitotic Index (%) (I_m)*
Patient A			
Biopsy 1 $I_{m(o)}$	6781	160	2·36
Biopsy 2 $I_{m(t)}$	9048	701	7·75
Patient B			
Biopsy 1 $I_{m(o)}$	8421	429	5·09
Biopsy 2 $I_{m(t)}$	15 288	2264	14·81

Table *Summary of cytokinetic data in patients A and B*

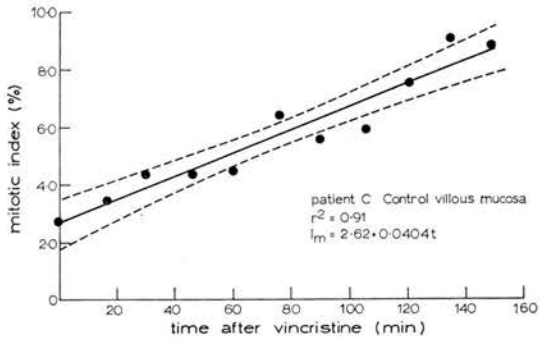

Fig 1a

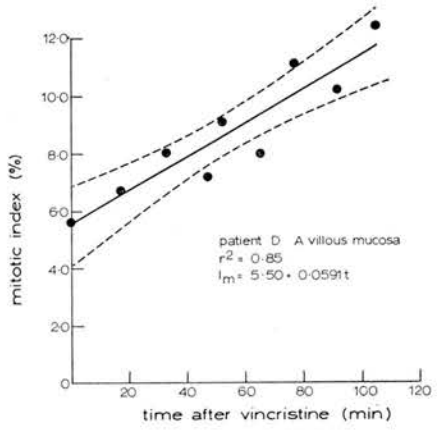

Fig 1b

Fig. *Metaphase accumulation lines for* (a) *patient C, and* (b) *patient D. In each case vincristine was administered at time 0, and the slopes of the regression lines represent the rate of accumulation of arrested metaphases in the intestinal mucosa. The dotted lines represent 95% confidence limits for the line.*

ances of untreated gluten-sensitive enteropathy on histological examination.

The metaphase accumulation graphs for patients C and D (see fig) show that there is no apparent lag in the onset of vincristine metaphase arrest, and that a good degree of linearity is present over the experimental period. The slopes of the lines did not differ significantly ($0{\cdot}05 < P < 0{\cdot}06$). $T_{c(a)}$ was 58 hr in patient C and 39 hr in patient D. The corresponding values for T_c (corrected for growth fraction) were 42 hr and 21 hr respectively. In patient C t_m was 1·10 hr and in patient D 1·55 hr ($P > 0{\cdot}05$).

Discussion

It is concluded that the cell cycle time in the crypt cells of the avillous mucosa in untreated gluten-sensitive enteropathy is about half that in control villous mucosa. In patients A and B the difference between the $T_{c(a)}$ values was found to be statistically significant; presumably the difference between the T_c values is also real. It was not possible to prove statistical significance between the $T_{c(a)}$ values of patients C and D. However, when the growth fraction is incorporated into the calculation, it can be seen that again there is a halving of the cell cycle time in the flat mucosa. In each case t_m is calculated from a quotient of mitotic indices; consequently because of the larger standard errors involved it is not possible to say whether a real difference exists in t_m values between the villous control and avillous flat mucosae.

The nature of the metaphase accumulation lines suggest that the assumptions made in the first experiment were probably justified. In experimental animals Wright, Morley, and Appleton (1972) have shown that the values for mitotic duration obtained by the use of equation 2 were in close agreement with an accurate technique involving measurement from a metaphase collection graph. All the equations in the present report assume steady state and they are easily modified to allow for exponential conditions; however, there is as yet no evidence that in the hyperproductive mucosal state found in flat mucosae steady state conditions do not apply.

The values given for cell cycle time in the control mucosae are in reasonable agreement with the values obtained by Lipkin, Bell, and Sherlock (1963) using labelling techniques with tritiated thymidine. Values for cell cycle time in flat mucosae have not been reported hitherto and all measurements of the proliferative state have been indirect (Wright *et al*, 1973) and largely dependent upon mitotic indices only. The T_c values given in this paper are direct measurements and have been corrected for changes in proliferating population, and consequently are free from the several criticisms which can be levelled against other measurements of proliferative status.

The implications of this study for the mucosal dynamic state in flat mucosae are several. The decrease in cell cycle time of crypt cells indicates that even if there were no increase in the proliferative population, cell production rate would be at least doubled. In a further paper (Wright *et al*, 1973) we shall demonstrate a threefold increase in the number of proliferating cells per crypt in gluten-sensitive enteropathy; this is despite the lowered growth fraction, and is a consequence of the three-dimensional increase in crypt size. Independent evidence is available which supports these observations; Croft, Loehry, and Creamer (1968) have demonstrated an increased cell loss (in terms of

nuclear DNA) in patients with the coeliac syndrome in relapse. Trier and Browning (1970), employing an elegant *in-vitro* technique using tritiated thymidine, reported increased labelling indices and cell migration rates in flat mucosa compared with control villous mucosae. Combined with the present observation of a markedly decreased cell cycle time, the existence of a hyperproductive mucosal state in untreated gluten-sensitive enteropathy (Booth, 1970) can be regarded as proven.

We are grateful to Professor A. G. Heppleston for continued encouragement and advice. Mrs Glynis Stones rendered skilled technical assistance and the work was supported by a grant from the North of England branch of the Cancer Campaign.

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Cell kinetics in flat (avillous) mucosa of the human small intestine

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Gut, 1973, **14**, 701-710

Cell kinetics in flat (avillous) mucosa of the human small intestine

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SUMMARY A new method for the analysis of small-intestinal crypt-cell kinetics using routine peroral diagnostic biopsies is described. Untreated patients with childhood and adult coeliac disease and adults with the gluten-sensitive enteropathy of dermatitis herpetiformis were studied, together with groups of adult and childhood controls. In the classical flat avillous mucosae the increase in crypt size was found to be three-dimensional. The number of proliferating cells per crypt was shown to be markedly increased, and an even greater rise in the crypt-cell production rate was demonstrated. A significant increase in the mitotic index was also confirmed in the avillous mucosae.

On the basis of these findings it is suggested that the characteristic crypt morphology in gluten-sensitive enteropathy can be explained as an adjustment to accommodate the expanded mass of proliferating and maturing cells necessary to support the augmented cell production rate. We may speculate that this in turn is a response to a pathologically rapid loss of cells from the mucosal surface.

With the introduction of peroral biopsy of the small bowel, morphological assessment of the mucosa became a routine investigatory procedure. It was soon confirmed (Bertalanffy and Nagy, 1961) that the rapid epithelial turnover already recognized in animals also occurred in the normal human small bowel. Despite early indications that a severe disturbance of mucosal dynamics was present in coeliac disease (Padykula, Strauss, Ladman, and Gardner, 1961), progress in characterizing this important aspect has been haltingly slow.

Indirect observations have certainly suggested that the rate of mucosal-cell loss in coeliac disease is greatly increased (Pink, Croft, and Creamer, 1970), and an obvious corollary is that the rate of cell production must be similarly enhanced. Padykula *et al* (1961) reported an increased mitotic index in coeliac disease, an observation confirmed by Yardley, Bayless, Norton, and Hendrix (1962). On the other hand Creamer (1962) found a low mitotic index in the crypts of coeliac disease compared with normal crypts, and in one patient demonstrated a slower cell migration rate. These findings were interpreted as indicating a prolonged turnover time with perhaps a defective maturation sequence.

Received for publication 16 May 1973.

Booth (1970) in a recent review considered that the bulk of the evidence was in favour of a hyperproliferative state in coeliac disease, and suggested that the characteristic changes in crypt morphology were a direct consequence of 'enteroblastic hyperplasia' compensating for excessive loss of surface enterocytes. This hypothesis, that the characteristic changes in crypt morphology result from an induced change in the functional activity of the crypts, can only be substantiated by detailed analysis of the organization of cell renewal in the flat avillous mucosae of coeliac disease. A factor which should also be taken into account and which may explain some previous discrepancies is the possible heterogeneity of coeliac disease. For example, is the kinetic disturbance similar in the childhood and adult syndromes, and what is the relation of these to the gluten-sensitive enteropathy of dermatitis herpetiformis?

Hitherto there has been a lack of safe techniques of sufficient sophistication to provide information about the more important proliferative parameters established in experimental animals. We have evolved a technique by which useful data can be obtained from routine peroral small bowel biopsies and have studied cell kinetics in patients with childhood and adult coeliac disease, in cases of dermatitis herpetiformis with a flat mucosa, and in appropriate controls.

Materials and Methods

BIOPSY SPECIMENS

All mucosal specimens studied were obtained by peroral biopsy of the duodeno-jejunal region using a modified Crosby capsule. Most biopsies were taken at about the middle of the day in an attempt to exclude diurnal variation of proliferative indices (Sigdestad, Banman, and Lesher, 1969). Biopsies were orientated under the stereomicroscope, spread on glass, and immediately fixed in 10% neutral buffered formol saline. To improve nuclear staining specimens were postfixed in a solution of mercuric chloride. Specimens were then embedded in paraffin wax and serially sectioned at a thickness of 3 μm. Sections for analysis were stained with Harris's haematoxylin.

PATIENTS

Two series of control patients were studied. The adult controls comprised 75 persons, males and females, over the age of 20. The childhood controls were 10 in number, all below 7 years of age. Inclusion in the control groups was dependent purely on the morphological appearance of the small bowel biopsy and was not related to the patient's clinical history. Such control biopsies were those showing finger-shaped or leaf-shaped villi on stereomicroscopy and an absence of pathological features on histological examination. Biopsies showing convolutions or protoconvolutions were excluded even in the absence of any significant histological abnormality. The patients with a flat avillous mucosa totalled 62, and comprised 13 patients with adult coeliac disease (ACD), 20 adult patients with dermatitis herpetiformis (DH), and 29 patients with childhood coeliac disease (CCD).

CRYPT ANALYSIS

In virtually all cases 30 crypts were selected for examination but in a few instances, because of poor orientation or inadequate size of the specimen, less than 30 crypts were available. Only complete axial crypt sections were analysed, ie, those in which the base, middle, and mouth of the crypt were all in the plane of section. Care was taken to ensure that each crypt was analysed only once. The counting techniques of Cairnie, Lamerton, and Steel (1965a) were used throughout. The identity of the sections was unknown to the observer. In each crypt the left hand column of cells was numbered, counting from the bottom upwards to the crypt-villus junction; this is referred to as the 'crypt column', illustrated in figure 1. The positions in the crypt column of cells in mitosis were recorded. A mitosis situated midway between the right and left crypt columns was counted as a half (Cairnie *et al*, 1965a).

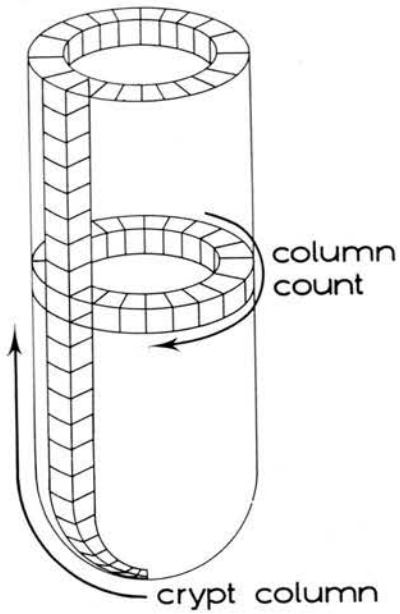

Fig 1 *Diagrammatic representation of the column count and crypt column.*

In any sample of small intestinal crypts both in man and in animals considerable variation is found in the height of the crypt columns (Cairnie *et al*, 1965a; Wright, Morley, and Appleton, 1972a and b) and in calculating the mitotic index as a function of cell position in the crypt column such variation should be taken into account. Plotting the variation in mitotic index with cell position gives a mitotic index distribution curve. Figure 2 illustrates the method of construction. In normal animals Cairnie and Bentley (1967) found differences in the distribution of tritiated thymidine-labelled cells in short and long crypts according to whether the positions of cells were taken relative to the top or bottom of the crypt; clearly the same argument applies to the distribution of mitoses.

In order to compensate for this variation, the data were processed by an ALGOL computer program run on an IBM 360/67 computer. The details of the method are given elsewhere (Wright *et al*, 1972a and b), but is based on the hypothesis that a crypt cell's location is best described not by its position relative to the top or bottom of the crypt but by its position relative to the total number of cells in the crypt column. Consequently each crypt is projected onto a large standard crypt for the calculation of the mitotic index at each cell position, and then onto a

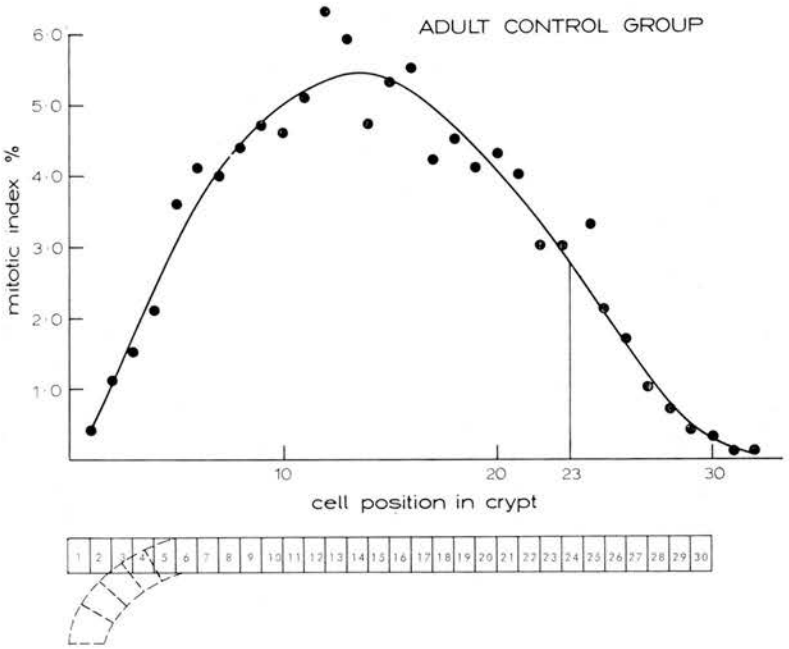

Fig 2 *The method of construction of a mitotic index distribution curve. The crypt column is shown horizontally to illustrate that the individual points on the graph represent mitotic indices at each cell position.*

crypt whose size is the mean for the group analysed. This manoeuvre reduces the difference in mitotic index distribution in long and short crypts from the same sample.

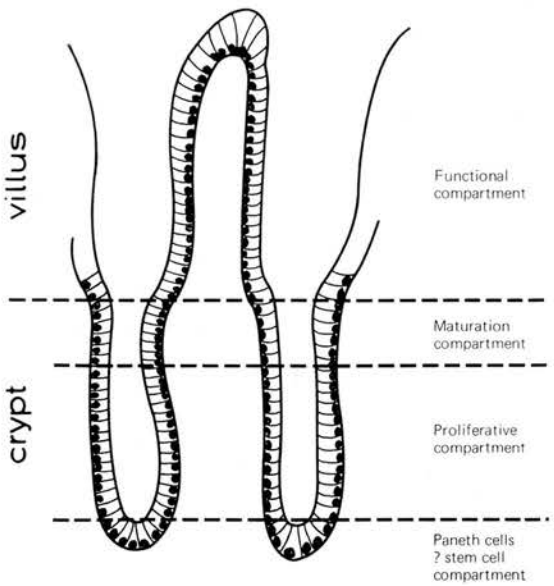

Fig 3 *Diagrammatic representation of the kinetic compartments in the small bowel mucosa.*

In the crypts two morphometric parameters were measured, and these are illustrated in figure 1. The length of the crypt column in terms of cell numbers was measured in the analysis of the axially sectioned crypts described above. Afterwards such material as remained in the blocks was reorientated and sectioned transversely through the crypts for measurement of the column count. The column count is the number of cells in a crypt cross section, and is equal to the number of crypt columns in the crypt. This measurement involved counts carried out on 100 crypt cross sections in each specimen analysed.

THE KINETIC COMPARTMENTS

The small bowel mucosa can be divided into several kinetic compartments (fig 3), each fulfilling a particular role in cell production. Most cell production occurs in the lower portion of the crypt, called the proliferative compartment. Moving upwards, cells lose the ability to divide and pass into the maturation compartment. From here differentiated cells move into the functional compartment on the surface of the villus. Recently a further compartment has been delineated, the stem-cell compartment, which is located at the base of the crypt and supplies cells to the proliferative compartment (Lamerton, 1972; Wright *et al*, 1972b).

The changes in the sizes of these compartments which occur in coeliac disease may be important in

understanding the evolution of the characteristic morphology. The method of analysis described in this paper allows comparative estimates of the proliferative and maturation compartment sizes in terms of the fractional length of the crypt column. Perhaps a more realistic measure of these compartments lies in the actual number of cells which they contain. Wimber and Lamerton (1963) have shown that the product of the column count and the crypt column gives a reliable estimate of the total crypt population. Knowledge of the fraction of the crypt occupied by a particular compartment then allows calculation of the total number of cells present in that compartment.

Inspection of fig 3 shows that a critically important region of the crypt is the transition zone from the proliferative to the maturation compartment, called the 'cut-off region' (Cairnie *et al*, 1965b). Changes in the position of this cut-off region will control the size of the proliferative compartment, and therefore the growth fraction, ie, the proportion of crypt cells actually dividing.

Results and Interpretation

The distribution in the height of the crypt columns is shown for the control groups in fig 4 and the mean values in table I. A slight increase in both column height and column count is apparent in the childhood controls compared with adults, and the estimate of the total crypt population obtained from these two parameters is similarly increased. The column height distributions for the flat mucosae of childhood and adult coeliac disease and dermatitis herpetiformis are shown in fig 5, and mean values of both morphometric parameters are also given in table I. In each of the three groups there is marked lengthening of the crypt column, and the lengths attained are not significantly different. However, the column counts in the adult coeliac disease group show a large increase over that in both childhood coeliac disease and dermatitis herpetiformis, the values in the latter two groups being not significantly different. All three groups show large increases in column count over the control values. The estimates of total crypt-cell populations likewise show a three- to fourfold increase compared with the controls. Crypt enlargement in coeliac disease is therefore three-dimensional, with the crypts in mucosae of adult coeliac disease containing the most cells.

	Crypt Column (cells)	*Column Count (cells)*	*Total Cells in Crypt*
Adult controls	31·9 ± 0·1	24·5 ± 0·2	780
Adult coeliac disease	74·1 ± 0·6	41·2 ± 0·3	3050
Dermatitis herpetiformis	75·7 ± 0·6	35·7 ± 0·3	2700
Childhood controls	34·4 ± 0·3	25·3 ± 0·2	870
Childhood coeliac disease	74·9 ± 0·6	35·3 ± 0·5	2640

Table I *Morphometric parameters in control and flat mucosae with standard errors*

In each group the mitotic index (I_m) was calculated for each cell position and mitotic index distribution curves are shown for the control groups in fig 6 and for the flat mucosae in figure 7. The adult control curve shows low I_m values in the lower cell positions rising from below 0·5% at cell position 1 to reach a peak of over 5% between positions 10-15. This peak is followed by a slow return to low values as the maturation compartment is reached.

The growth fraction can be calculated approximately from the mitotic index distribution curve. It is evident that a growth fraction calculated for the whole crypt represents the fraction of the crypt population that is in the proliferative compartment. This

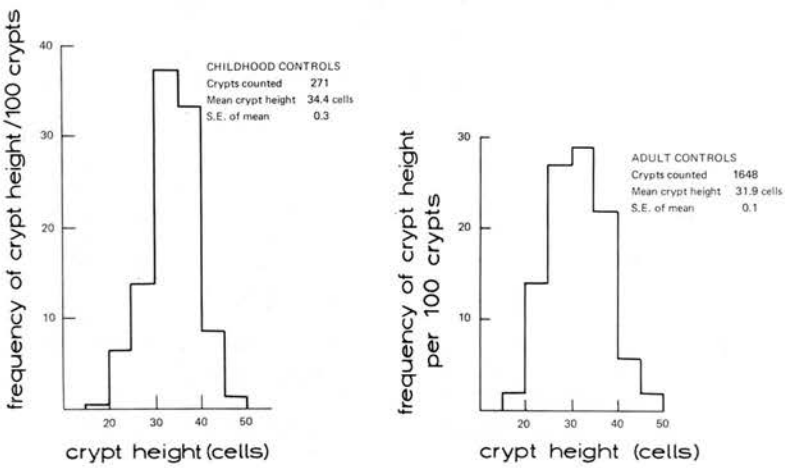

Fig 4 *The distribution in the height of the crypt columns in the control groups.*

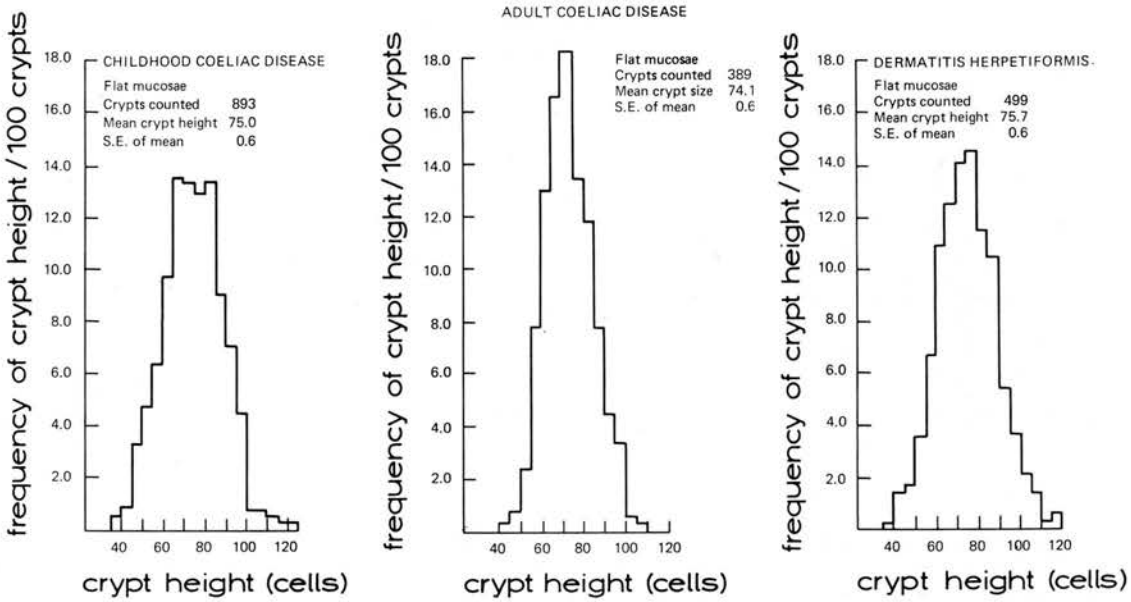

Fig 5 *The distribution in the height of the crypt columns in the flat mucosae.*

is the region over which mitoses are observed and it may be considered to end at approximately the cell position where the I_m falls to 50% of its maximum or peak value (Cleaver, 1967). The maturation compartment then becomes those cells above the cell position where the I_m falls to 50% of the maximum value.

In the mitotic index distribution curve for the adult controls the 50% peak value is found at cell position 23, and the growth fraction (I_p) for the whole crypt may be taken as the ratio of the proliferative compartment size of 23 cells to the total cells in the crypt column, ie, 23/32, or 0·72. The childhood controls show an I_m in the lower cell positions greater than in the adult controls. This bottom end of the crypt is the portion occupied by the stem cells and these findings may reflect kinetic differences in this compartment between these two groups. The curve then rises to reach a slightly higher peak of over 6% between cell positions 10 and 15, before falling again as in the adult controls. The 50% peak value is again at cell position 23, but since the crypts are longer the growth fraction is 23/34 or 0·68, slightly lower than in the adult controls.

All the flat mucosae show large differences in I_m distribution compared with the controls. In adult coeliac disease, high values of around 2% are apparent in the stem-cell compartment followed by a sharp rise to reach a maximum I_m of 11·5% at between cell positions 15 and 20. A steep fall then occurs, with the 50% peak value at position 41, giving an I_p estimate of 0·55. The I_m distribution curve of dermatitis herpetiformis shows a similar configuration to that of adult coeliac disease with several minor differences. I_m in the stem cell compartment is lower even than in the adult controls. The

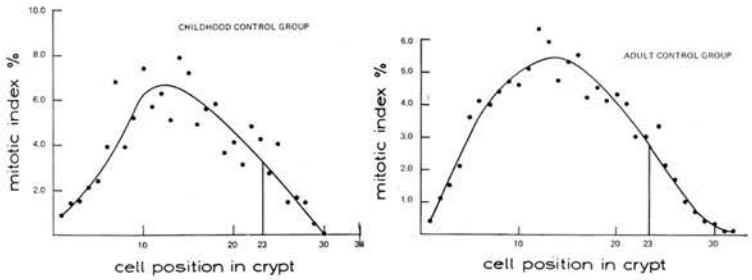

Fig 6 *Mitotic index distribution curves for the control groups.*

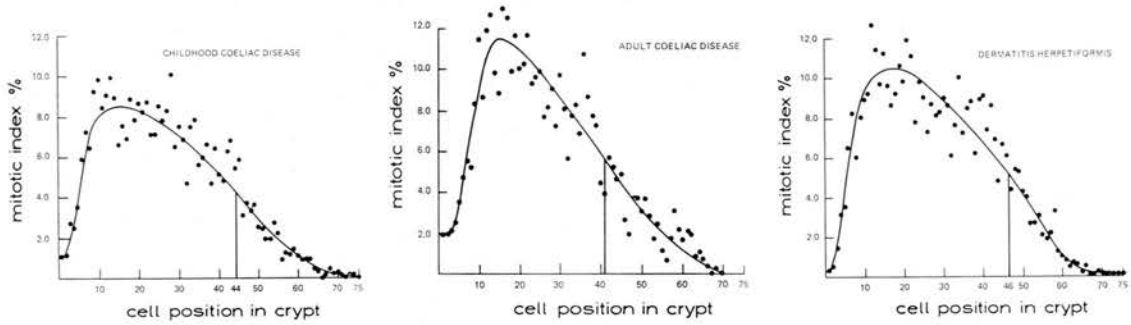

Fig 7 *Mitotic index distribution curves for the flat mucosae.*

peak value is somewhat less than in adult coeliac disease, realizing 10·0% between positions 10 and 23. The trailing edge of the curve is less steep than in adult coeliac disease, with a 50% peak value of 46 giving an I_p estimate of 0·60.

Childhood coeliac disease shows an I_m in the stem cell compartment similar to the childhood controls. However a sharp rise then occurs to reach a maximum I_m of over 8% between cell positions 12 and 23. The 50% peak value is 44 and the I_p 0·59. As mentioned above, a growth fraction calculated for the whole crypt represents the fraction of the crypt population that is in the proliferative compartment. The remaining cells can be considered to have left the cell cycle and to be occupying the maturation compartment. The compartment sizes measured from the I_m distribution curves are summarized in table II. Although minor differences are apparent between the three groups of flat mucosae an absolute increase in proliferative compartment size (in terms of cells) is evident compared with the control values. On the other hand there is a relative reduction in proliferative compartment size compared with the controls when the parameter is expressed as a fraction of the total crypt column. In the case of the maturation compartment in each group both an absolute and a relative increase in size is found in the flat mucosae compared with the control values.

From estimates of the growth fraction, and knowing the number of cells per crypt, it is possible to arrive at a value for the total number of proliferating cells per crypt (table II). As compared with controls, a threefold increase in proliferating cells per crypt can be demonstrated in adult coeliac disease and dermatitis herpetiformis, while in childhood coeliac disease the increase is a little less than threefold. Despite the finding of a relative reduction in proliferative compartment size as measured from I_m distribution curves in the coeliac groups, the three-dimensional increase in crypt size nevertheless entails this large increase in proliferating cells per crypt. It should be noted that although the crypt population in adult coeliac disease is greater than in dermatitis herpetiformis the number of proliferating cells per crypt is similar because of the larger I_p in dermatitis herpetiformis.

The value obtained for the I_m expressed as a percentage of all crypt cells counted constitutes the crude mitotic index (table III). Each flat mucosa group shows a significant increase over the value in its respective control group. The mitotic index is directly dependent upon the growth fraction, and particularly in circumstances such as the present, when variation in I_p has been shown between the contrasted groups, should be expressed as a percentage of the proliferating population. When this is

	Proliferative Compartment Column Size (cells)	*Maturation Compartment Column Size (cells)*	*Growth Fraction*	*Total Number of Proliferating Cells per Crypt*	*Total Number of Maturing Cells per Crypt*
Adult controls	23 (72%)	9 (28%)	0·72	560	220
Adult coeliac disease	41 (55%)	33 (45%)	0·55	1680	1370
Dermatitis herpetiformis	46 (60%)	30 (40%)	0·60	1620	1080
Childhood controls	23 (68%)	11 (32%)	0·68	590	280
Childhood coeliac disease	44 (59%)	31 (41%)	0·59	1560	1080

Table II *Compartment sizes and proliferating populations in control and flat mucosae*[1]

[1]In this table the proliferative compartment includes the stem cells.

	Crude Mitotic Index (%)	*Corrected Mitotic Index (%)*
Adult controls	3·05	4·23
Adult coeliac disease	5·22	9·49
Dermatitis herpetiformis	5·46	9·10
Childhood controls	3·47	5·10
Childhood coeliac disease	4·58	7·77

Table III *Mitotic indices in control and flat mucosae*[1]

[1]See text.

done, the 'corrected' mitotic indices indicate even larger differences between the flat mucosa groups and their controls; in the case of adult coeliac disease and dermatitis herpetiformis the corrected mitotic index is more than double the adult control value. Both these conditions show significantly higher mitotic indices than childhood coeliac disease ($P < 0{\cdot}001$), but there is no significant difference between adult coeliac disease and dermatitis herpetiformis.

If it is assumed that the duration of mitosis does not differ in the groups studied, and if we further assume that this parameter is one hr, then comparative values for the rate of cell production, the migration rate, and the transit times can be calculated. This assumption is defended in the discussion, and has been explored in subsequent investigations (Wright, Watson, Morley, Appleton, Marks, and Douglas, 1973).

The birth rate (k_b), or rate of cell production by cell division alone, can be calculated from the ratio of the mitotic index (I_m) to the mitotic duration (t_m).

$$k_b = \frac{I_m}{t_m} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

If the birth rate is calculated for each cell position and these values are then summated, a cumulative birth rate curve is obtained (Cairnie *et al*, 1965a; Wright *et al*, 1972a and b) and the efflux past any cell position is equal to the sum of the birth rates up to that position (Cleaver, 1967). Furthermore, the maximum birth rate attained is equal to the total efflux from the top of the crypt column, and is also equal to the migration rate at any point above which cell proliferation has ceased, since the maturation compartment is a simple transit compartment.

Cumulative birth rate curves are shown for the controls in fig 8 and for the flat mucosae in figure 9. Values for the maximum birth rate attained, or in other words, the migration rate, are shown in table IV. In each coeliac group there is a three- to fourfold increase in migration rate compared with control values, the increase being most prominent in dermatitis herpetiformis and adult coeliac disease. The product of the migration rate and the column count gives an estimate of cell production rate per crypt. (table IV). The adult control group shows a cell production rate of 25 cells per crypt per hour, increased to some 150 cells per hour in adult coeliac disease and hermatitis herpetiformis. The increase in childhood coeliac disease is smaller but nonetheless considerable.

	Migration Rate (cell positions/hour)	*Cell Production Rate (cells/crypt/hour)*
Adult controls	1·0	25
Adult coeliac disease	3·8	155
Dermatitis herpetiformis	4·0	143
Childhood controls	1·2	29
Childhood coeliac disease	3·4	120

Table IV *Crypt efflux in control and flat mucosae, assuming a mitotic duration of one hr in all instances*

The transit times through the various compartments can be estimated from the above cumulative birth rate curves by taking the reciprocal of the

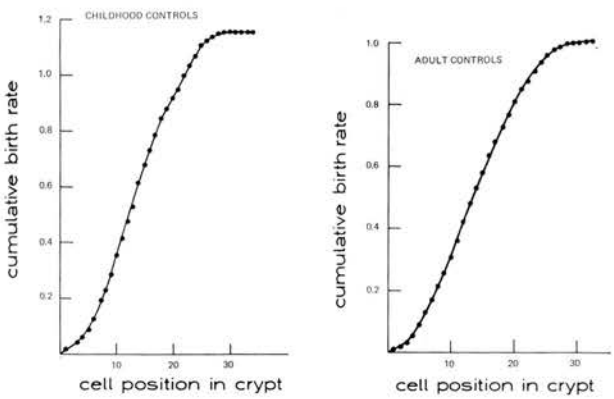

Fig 8 *Cumulative birth rate curves for the control groups.*

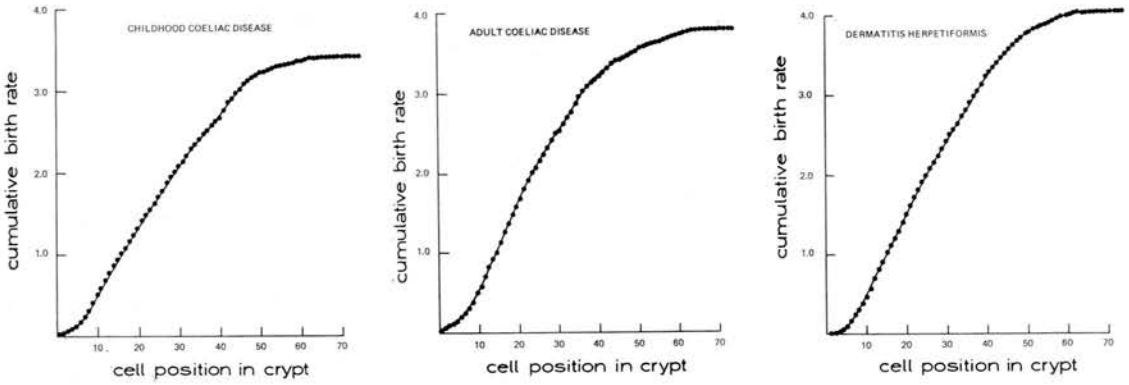

Fig 9 *Cumulative birth rate curves for the flat mucosae.*

cumulative birth rate at each cell position and summating these values over the relevant portion of the crypt (table V). The problem of defining the size of the stem-cell compartment has been considered by several investigators (Cleaver, 1967; Wright *et al*, 1972a and b). In this investigation an arbitrary delineation of this compartment has been made and stem-cells are regarded as occupying those cell positions with a transit time in excess of 10 hours. In effect this places the first five cell positions of the control groups within this compartment, and also the first five cell positions of the flat mucosa groups. Proliferative-compartment transit time is slightly decreased in both adult coeliac disease and dermatitis herpetiformis while maturation-compartment transit time is similar in both. This similarity in proliferative and maturation compartment transit times between the control and coeliac groups is a reflection of the increased migration rate in the elongated crypts of the flat mucosae. In the childhood controls the transit times are somewhat faster than in the adults, but because of the increased migration rate in childhood coeliac disease, transit times in that and in the childhood controls are similar.

Discussion

The conclusions made possible by this study permit a formalization of current thought on the mechanism of evolution of a flat mucosa in coeliac disease. In the flat mucosae of the three groups of patients with gluten-sensitive enteropathy the crypts have been shown to be increased not only in length but also in girth. There is a large increase in the total crypt population, and in particular the number of proliferating cells per crypt is increased threefold. The total number of maturing cells is even more markedly increased, for example, from 220 in the adult control group to 1370 in adult coeliac disease, a sixfold increase (see table II). This great increase in maturing cells can be thought of as a natural consequence of the increased output from the proliferative compartment, an output which is increased five to sixfold. This enhanced output of cells must be accommodated during maturation in a much enlarged maturation compartment.

The altered crypt morphology in gluten-sensitive enteropathy is therefore a response to the need to accommodate a greatly increased mass of proliferating and maturing cells. These in turn are needed to keep pace with the increased rate of surface cell loss which has been shown in this condition (Pink *et al*, 1970), and a sequence of compensatory reactions in the crypt can be envisaged. A moderate loss could be made good by upward movement of the 'cut-off position' leading to an increase in the growth fraction

	Transit Times (hours)				
	Stem	*Proliferative*	*Maturation*	*Total*	*(Total-Stem)*
Adult controls	379	39	8	427	47
Adult coeliac disease	124	36	9	170	45
Dermatitis herpetiformis	540	33	7	581	40
Childhood controls	218	34	8	261	43
Childhood coeliac disease	196	34	8	239	43

Table V *Transit times for control and flat mucosae assuming a mitotic duration of one hr in all instances*

(Wright *et al*, 1972a). But if this response were insufficient to meet a greater loss the proliferating cell mass could be further increased only by crypt enlargement, and this evidently occurs in three dimensions, with a resulting increase in length and girth of the crypts.

The relatively large numbers of patients analysed in each group, together with the standardized counting techniques, makes the values obtained for the mitotic indices likely to be particularly valuable statistically. It has been established that there is a significant increase in the mitotic index in the flat mucosa of gluten-sensitive enteropathy in accord with the observations of Padykula *et al* (1961) and Yardley *et al* (1963). There are, however, certain problems involved in the interpretation of a crude mitotic index, which by itself should not be regarded as a definitive index of proliferative state. It is essential to relate the mitotic index to the proliferating cells only, as the existence of a significant proportion of non-dividing cells will lead to an underestimate of proliferative activity; this is especially important when, as in the present study, comparisons are drawn between cell populations with differing compartmental sizes. When the mitotic index is expressed as a percentage of the proliferating cells only, the increase in the flat mucosa groups then becomes much larger.

The relationship of changes in mitotic index to the rate of cell division (mitotic rate) can be evaluated only if the mitotic duration is known. The increased mitotic indices in the flat mucosa groups reflect an increased rate of cell division only if there is no change in mitotic duration. In the present paper we have assumed that there is no significant change in mitotic duration, and that this parameter is one hr in all groups. Wright, Watson, Morley, Appleton, Marks, and Douglas (1973) have shown that the mitotic duration in normal human jejunum is about one hr, and using this value we have obtained a migration rate of 1·0 cell positions/hr in the adult controls, which agrees exactly with the value of Lipkin (1965), achieved by direct observation of cells labelled with tritiated thymidine. Wright *et al* (1973) have shown that the mitotic duration in flat mucosae is also close to one hour.

It is therefore apparent that crypt-cell migration rate is increased in flat mucosae and this, together with the greater number of crypt columns, indicates a five or sixfold increase in cell production rate per crypt. A similar increase has been demonstrated indirectly by measuring the DNA content of intestinal washings (Pink *et al*, 1970) but a direct comparison would involve estimation of any changes in actual numbers of crypts in the entire small bowel in coeliac disease. The present data further suggest that differences exist in mucosal kinetics in the several gluten-sensitive enteropathy syndromes studied. It would appear that the increase in cell production rate is smaller in childhood coeliac disease than in the two adult groups; between adult coeliac disease and dermatitis herpetiformis there seems to be little difference. Our study has shown some minor changes in the mitotic index of the stem cell compartments and in the sizes of the other kinetic compartments. It should, however, be noted that the increases in cell production rate are very similar.

Corroborative evidence for these conclusions has come from the work of Trier and Browning (1970); studying small bowel mucosa maintained in organ culture, they demonstrated that in biopsies from patients with adult coeliac disease increased numbers of crypt cells became labelled on exposure to tritiated thymidine. An increased rate of cell migration was also shown. However, short-term kinetic studies performed in organ culture may be complicated by the apparent dissociation of rates of entry into mitosis and into DNA synthesis, possibly as a consequence of explantation (Simnett, 1971).

Some workers have suggested that one source of difficulty inherent in any morphometric study of small bowel mucosa is the definition of the crypt-villus junction. In practice we have not found this to be important, and in any event the large number of crypts analysed compensates for any minor variation in interpretation. A further difficulty is in the choice of controls. A random normal population cannot readily be defined (Baker, 1972) and obviously could not be biopsied. What have been compared here are basically the kinetic differences between villous and avillous mucosae.

To summarize our findings, therefore, we conclude that in the flat mucosa of coeliac disease there is a large increase in cell production rate per crypt. There is also a three-dimensional increase in crypt size, and we propose that these morphological appearances are the result of a hyper-proliferative mucosal adaptation to the need for an augmented cell production rate, namely, the necessity to accommodate the required large numbers of proliferating cells.

There is in these results an apparent anomaly from which a prediction can be made. It will be noted from tables IV and II that in adult coeliac disease there is a sixfold increase in cell production rate, but only a threefold increase in the numbers of proliferating cells. The cells within the proliferative compartment may be dividing faster; that is, there may be a decrease in the cell cycle time, T_c (the interval between two succeeding divisions of a cell). The mitotic index is almost doubled in adult coeliac disease (table III), and this is related to the cell cycle time thus:

$$I_m = \frac{t_m}{T_c} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The increase in mitotic index could be due to a marked prolongation in the duration of mitosis; we consider this unlikely and anticipate instead a reduction in the cell cycle time in the flat mucosa of gluten-sensitive enteropathy.

We are grateful to Professor A. G. Heppleston for continued encouragement and advice. Miss E. Robinson rendered skilled technical assistance. This work was supported by the North of England branch of the Cancer Campaign.

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Virchows Arch. A Path. Anat. and Histol. 364, 311—323 (1974)
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The Measurement of Cell Production Rates in the Crypts of Lieberkuhn

An Experimental and Clinical Study

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Received July 8, 1974

Summary. A method is described of assessing the proliferative state in the small bowel mucosa employing the metaphase-arresting (stathmokinetic) properties of vincristine, applicable to both animals and man. In the rat a value for the migration rate of 1.78 cell positions per hour was obtained by the stathmokinetic method, in agreement with a further measurement of the migration rate made using tritiated thymidine. Values for the transit time through the crypt (34 hours) and for the cell production rate (39 cells per crypt per hour), are in good agreement with previously published values.

A control patient and a patient with the flat mucosa of gluten-sensitive enteropathy were also studied by the vincristine technique. The migration and cell production rates were markedly increased in the flat mucosae. A hyperproductive proliferative state is therefore confirmed in the flat mucosa of untreated gluten-sensitive enteropathy.

The results show the vincristine technique to be eminently applicable to problems involving intestinal cell kinetics.

Introduction

In recent years the detailed work of Lipkin (1965) has allowed estimation of the time parameters of the cell cycle in the human small bowel by the use of techniques involving tritiated thymidine in individuals with disseminated malignancies; unfortunately such techniques are too hazardous for routine use. In view of the debate concerning the mucosal proliferative state in the gluten-sensitive enteropathies (Creamer, 1962; Booth, 1970), and because of the current interest in the influence of irradiation and cycle specific chemotherapeutic agents on crypt cell kinetics (Skipper and Schabel, 1971; Lamerton, 1972) it has become necessary to evolve a simple technique by which mucosal dynamics can be studied in routine peroral biopsies.

The most satisfactory single proliferative parameter which has emerged from recent work on small bowel mucosal kinetics is the cell production rate per crypt (Clarke, 1971). This measurement is the resultant of many interrelated kinetic parameters; for example changes in growth fraction or in cell cycle time are reflected in the cell production rate. In the rat, Clarke has measured cell production rates by a stathmokinetic technique involving colcemid. We have evolved an alternative method of measuring cell production rate; a method which also yields information on several other important proliferative parameters. In this paper we substantiate the validity of the method experimentally and demonstrate its clinical applicability.

Materials and Methods

I. Experimental Material

Male albino Wistar rats aged 3 months and weighing 150–200 g were used throughout. All injection procedures were carried out at 09.00 h. Twelve animals were given 0.5 μCi of tritiated thymidine (^{3}HTdr) per g body weight by intraperitoneal injection. The ^{3}HTdr was obtained from the Radiochemical Centre, Amersham, England, and was of specific activity 5.0 Ci/mM. The concentration used was 100 μCi/ml. Samples of small bowel were taken from the region immediately distal to the ligament of Treitz, and would thus be described as upper jejunum. The tissue was fixed in Carnoy's fluid for 6 h, paraffin embedded, and serial transverse sections were cut at a thickness of 3 μm. Autoradiographs were prepared as previously described (Wright, Morley and Appleton, 1972a), using Kodak AR 10 stripping plates and an exposure time of 28 days.

In each animal 100 crypts were selected. Only complete axial crypt sections were chosen i.e. those in which the base, middle and mouth of the crypt were all present in the plane of section. Care was taken to ensure that each crypt was analysed only once. The counting techniques of Cairnie, Lamerton and Steel (1965a) were used throughout. The identity of the sections was unknown to the observer. In each crypt the left hand column of cells was numbered, counting from the bottom upwards to the crypt-villus junction, and the cell positions of labelled nuclei were recorded. During crypt analysis the number of cells present in a column was also recorded; this is the *crypt column*, and a further morphometric parameter was measured, the *column count* (Wright, Watson, Morley, Appleton and Marks, 1973). This was carried out in three untreated animals by cutting sections tangential to the bowel lumen so that crypts were cut in transverse section. In each animal the column count was estimated from counts on 100 approximately circular crypt cross sections.

II. Clinical Material

Patient A was a male aged 61, with a clinical history of rosacea. On day 1 a modified Crosby capsule was passed, and a mucosal biopsy of the duodeno-jejunal region was obtained in the usual way at 13.30 h. On day 2 the capsule was again passed and at 10.45 h was adjudged radiologically to be in the same position as on the previous day. At 11.00 h vincristine sulphate was rapidly infused intravenously in a dose of 0.045 mg/kg body weight. At 13.30 h precisely the capsule was fired and a mucosal biopsy obtained. Care was taken to ensure that no appreciable movement of the capsule occurred during the stathmokinetic period following vincristine injection.

Patient B was a male aged 57, with a ten year history of dermatitis herpetiformis. Two mucosal biopsies were obtained following the same protocol as that outlined above, again ensuring that the duration of the stathmokinetic period was exactly 2.5 h.

The biopsies were spread flat under the stereomicroscope and fixed for 24 h in neutral buffered formol saline and for a further 24 h in $HgCl_2$-formol solution. Serial paraffin sections were cut at a thickness of 3 μm and stained with Harris's haematoxylin. In patients A and B quantitative analysis was carried out in the manner already described and the column count was obtained as before from sections cut tangentially to the bowel lumen.

Both patients received full information concerning the investigative procedure and informed consent was obtained in each case; careful follow up has revealed no evidence of harmful effects due to vincristine.

In any sample of small intestinal crypts considerable variation is found in the height of the crypt columns; in calculating the mitotic index (or labelling index) as a function of cell position such variation should be taken into account. Cairnie and Bentley (1967) found differences in the distribution of labelled cells in short and long crypts of normal animals depending on whether the positions of cells were taken relative to the top or bottom of the crypt; clearly the same argument applies to the distribution of mitoses. For this reason we have analysed the data by a

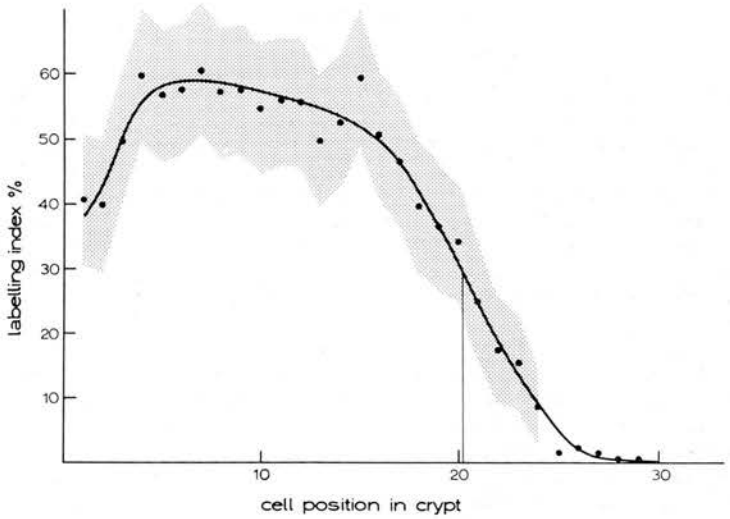

Fig. 1. Labelling index distribution curve for the 3 month old male Wistar rat. The shaded area indicates 95% confidence limits for the points, and the perpendicular shows the cell positions of the 50% peak value

modified version of the method designed by Cairnie and Bentley (1967), subjecting it to an ALGOL program run on an IBM 360/67 computer. Details of this procedure, and the method employed for the construction of *labelling and mitoitc index distribution curves*, are given elsewhere (Wright *et al.*, 1972a).

A nucleus was regarded as being labelled if 5 or more grains were localised over it (Wright, 1971). The morphological criteria used for the recognition of the various mitotic stages were similar to those of Clarke (1970).

Tannock (1967) has indicated that an important geometrical factor is involved in the measurement of the crypt mitotic index from tissue sections. Nuclei in mitosis are out of line with the interphase nuclei of the remainder of the crypt column, and are found to be nearer the crypt axis. This spatial arrangement leads to an overestimate of the mitotic index. Tannock therefore proposed a constant, a/b (where a is the radial position of mitoses in the crypt, and b is the radius of the crypt lumen), by which the mitotic index should be multiplied in order to correct for this error. Sections cut tangentially to the bowel were therefore examined. In the rat a value of the ratio a/b was obtained by measurement of 210 approximately circular crypt sections containing metaphases, using a calibrated eyepiece graticule. In patient A we measured 102, and in patient B 106 crypt cross sections.

Results and Interpretation

I. ^{3}HTdr Studies in Rats

In Fig. 1 is shown the labelling index (I_L) plotted as a function of the cell position in the crypt for two animals killed 1 h after ^{3}HTdr administration. Initially, at the base of the crypt lower indices are found, for example 40% at

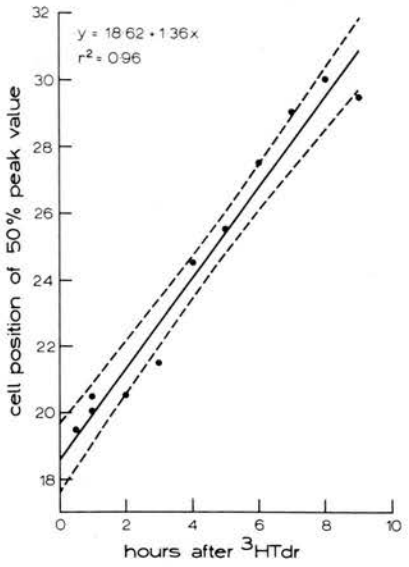

Fig. 2. Movement of the 50% peak value with time after injection of ^{3}HTdr. The solid line was fitted by linear regression analysis, and the dotted lines indicate 95% confidence limits for the line. The slope is then a measure of the migration rate

cell position 1. Cells in this portion of the crypt have previously been shown to have lower proliferative indices (Cairnie *et al.*, 1965a) and Wright, Morley and Appleton (1972b) have demonstrated a prolonged cell cycle time in these cells. They may well constitute a 'stem-cell compartment' for the contiguous proliferative compartment (Lamerton, 1972). A rapid increase in I_L then occurs to reach a maximum value of 60% at cell position 7. A steady decrease then follows, and very low indices are reached by cell position 25.

The growth fraction, or proliferating pool (I_p) calculated for the whole crypt represents the fraction of the crypt population that is in the proliferative compartment. The fraction of the crypt occupied exclusively by the *proliferative compartment* is the region over which labelled or mitotic cells are observed, and this region was considered to end approximately at the cell position where the I_L fell to 50% of the maximum or peak value (Cleaver, 1967). This was found to be at cell position 20. The I_p is equal to the ratio of this to the number of cells in the crypt column, i.e. 20/33, or 0.61. That is to say 61% of the crypt is occupied by the *proliferative compartment*, and the remainder by the maturation *compartment*, composed of differentiating cells. This compares well with an I_p value of 0.62 calculated from the duration of cell cycle phases and the labelling index (Wright, Aldewachi, Appleton and Watson, in preparation).

By taking the cell position of the 50% peak value as a reference point an estimate can be obtained of the cell migration rate. I_L distribution curves were constructed for each animal killed up to 9 h after ^{3}HTdr administration. After 9 h labelled cells began to appear on the villi. Fig. 2 shows the movement of

the 50% peak value with time after ^{3}HTdr administration. The two dotted lines are 95% confidence limits for the line. The slope of the line gives the cell migration rate expressed in cell positions traversed per h, and was equal to 1.36 ± 0.19 (95% confidence limits).

In a previous study (Wright *et al.*, 1972), we described a technique employing vincristine in which cumulative birth rate curves were constructed for the rat jejunal crypt from accurate values for the mitotic duration at each cell position. This method gives a cell velocity of 1.78 cell positions per h, in reasonable agreement with the value given by the ^{3}HTdr technique described above.

Table 1. Compartmental sizes and proliferative populations in the rat jejunal mucosa

Crypt column (cells)	Column count (cells)	Crypt population (cells)	Growth fraction	Number of proliferating cells
32.9 ± 0.2	22.3 ± 0.3[a]	734	0.61	448

[a] Standard error.

Table 1 shows the morphometric parameters obtained in the rat. The product of the crypt column and the column count gives an estimate of the total crypt population (Wimber and Lamerton, 1963; Cairnie and Bentley, 1967), and as calculated from the present data the crypt population is 734 cells. The growth fraction has been derived from the I_L distribution curve, and the product of the I_p and the total crypt population should give an estimate of the total number of proliferating cells per crypt. This is 448; the remaining 286 cells are differentiating cells occupying the maturation compartment.

A further kinetic parameter which can be calculated from these cummulative birth rate curves is the transit time (T) through each compartment. The transit time past any particular cell position is equal to the reciprocal of the birth rate at that cell position. If the compartment sizes are known, then calculation of their respective transit times can be found by summation. These are shown in Table 2. The crypt transit time (exclusive of 'stem cells') is 34 h. Assuming the first three cell positions to be 'stem cells' for the proliferative compartment (see discussion) the transit time for the proliferative compartment is 26 h, and for the maturation compartment 8 h.

Table 2. Flux parameters in the rat jejunal mucosa

Migration rate (cell positions per h)	Crypt cell production rate (cells/crypt/h)	$T_{\text{prolif.}}$ (h)	$T_{\text{mat.}}$ (h)	$T_{\text{prolif.+mat.}}$ (h)
1.78[a] 1.36[b]	39	26.1	7.9	34.0

[a] From cumulative birth rate curve.
[b] From movement of 50% peaks after labelling with ^3HTdr.

As already explained, the limiting value of the cumulative birth rate curve is equal to the efflux for a single crypt column, and in effect represents the rate of cell production for that column. If this value is multiplied by the total number of columns in the crypt (the column count), then a value for total cell production rate per crypt is obtained. This is equal to 39 cells per crypt per hour (Table 2), and is in good agreement with the value of 35 cells per crypt per hour obtained from microdissected crypts by Clarke (1971), also for the Wistar rat. Wright *et al.* (in preparation) also studying microdissected and squashed crypts, calculated values for crypt cell production rate of about 34 cells per crypt per h from measurements of the durations of the phases of the cell cycle and of the growth fraction. The present value at 39 may be slightly higher because sections have been employed (Clarke, 1968).

II. Vincristine Studies in Humans

The biopsies from patient A showed finger-shaped and narrow leaf shaped villi only, and histological examination showed no notable abnormality. Patient B showed a flat mucosa with a cobblestoned appearance in both biopsies, and the histological appearances were those of total villous atrophy. In each case the post-vincristine sample showed no evidence of anaphase escape, i.e. no anaphases were detected, and metaphase blockade was assumed to be complete.

In Table 3 are shown the morphometric parameters for the two cases, and it is evident that in addition to the well known lengthening of the crypts in flat mucosae there is also a considerable increase in the girth of the crypts. In flat mucosae there is clearly a *three-dimensional increase* in crypt size (Wright *et al.*, 1973), and this is reflected in the value obtained for the crypt population, an almost three-fold increase.

The I_m distribution curve constructed from the pre-vincristine sample from patient A is shown in Fig. 3a. The I_m rises from very low values of below 0.5% in the basal cell positions to a peak of nearly 4.0% at cell position 9; values above 3.0% are found thereafter until cell position 23, where there is a rapid fall to low values. As mentioned above, Cleaver (1967) considers that the proliferative compartment is that region where labelled *or mitotic* cells are observed, and here we are obliged to measure I_p from I_m distribution curves. As would be expected on the basis of the slow cut-off model (Cairnie, Lamerton and Steele, 1965b), this method gives higher values than those obtained from I_L distribution curve; however, since the I_m distribution curve is used throughout the human studies, direct comparison can be made. The 50% peak value is at cell position 26, and the I_p is therefore 0.81. The I_m distribution curve for the post-vincristine sample is shown in Fig. 3b, and although considerable increases are evident in individual I_m values at each cell position, it can be seen that there is no apparent upward displacement of the 50% peak values.

The I_m distribution curve for the pre-vincristine sample from patient B is shown in Fig. 3c. Again low values are evident in the lower cell positions, but a rapid rise to a higher peak value of over 10% between cell positions 11 and 22 then occurs, and is followed by a steady fall to low I_m values again towards the top of the crypt. The 50% peak value is at cell position 40 and this gives an I_p

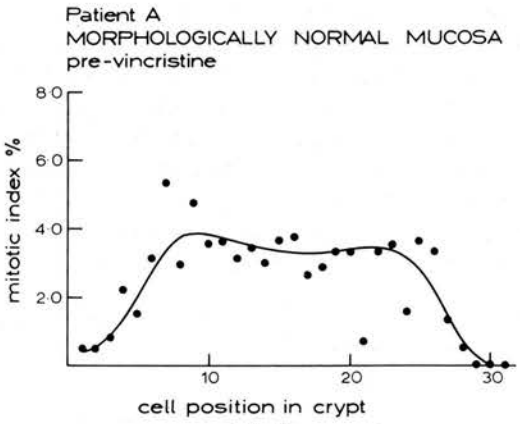

Fig. 3a

Fig. 3a—d. Mitotic index distribution curves, 3 (a) for the previncristine, and 3 (b) for the post-vincristine biopsies from patient A. 3 (c) and 3 (d) show the mitotic index distribution curves for the pre and postvincristine samples respectively for patient B. Note the increase in I_m at each position in the postvincristine samples, but without movement of the 50% peak value. The shaded areas indicate 95% confidence limits for the points

Table 3. Morphometric parameters in human small bowel mucosa; compartment sizes and proliferating populations in human small bowel mucosa

	Crypt column (cells)	Column count (cells)	Crypt population (cells)	Growth fraction	Number of proliferating cells/crypt
Patient A (normal villous mucosa)	31.9 ± 0.2	22.3 ± 0.6	711	0.81	576
Patient B (flat avillous mucosa)	67.0 ± 0.5	30.4 ± 0.5	2036	0.60	1222

	Proliferative compartment size (cells)[a]	Maturation compartment size (cells)[a]	Mitotic index (%)
Patient A	26 (81%)	7 (19%)	2.36
Patient B	40 (60%)	37 (40%)	5.09

[a] Measured from the previncristine I_m distribution curves.

value of 0.60. The I_m distribution curve obtained from the post-vincristine sample is shown in Fig. 3d; as expected there are considerable increases in values of I_m at individual cell positions; but again, there has been no apparent upward movement of the 50% peak values during the period of metaphase arrest. This demonstrates that vincristine effectively inhibits cell division and thus cell migration.

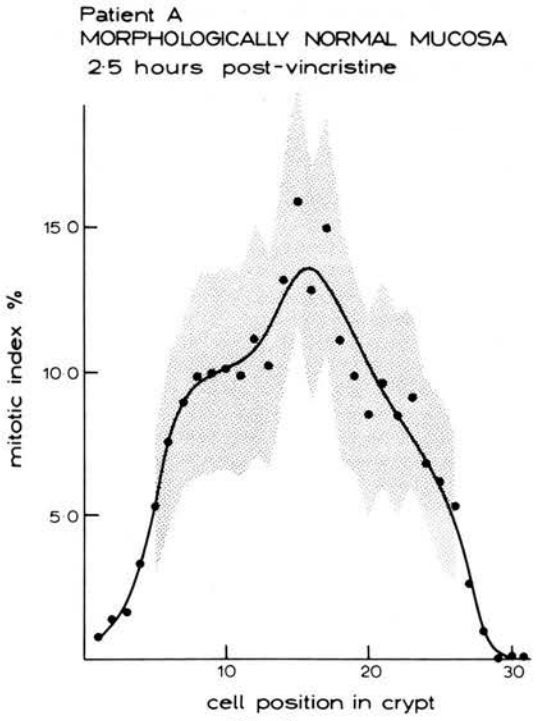
Patient A
MORPHOLOGICALLY NORMAL MUCOSA
2·5 hours post-vincristine
mitotic index %
15·0
10·0
5·0
10
20
30
cell position in crypt

Fig. 3b

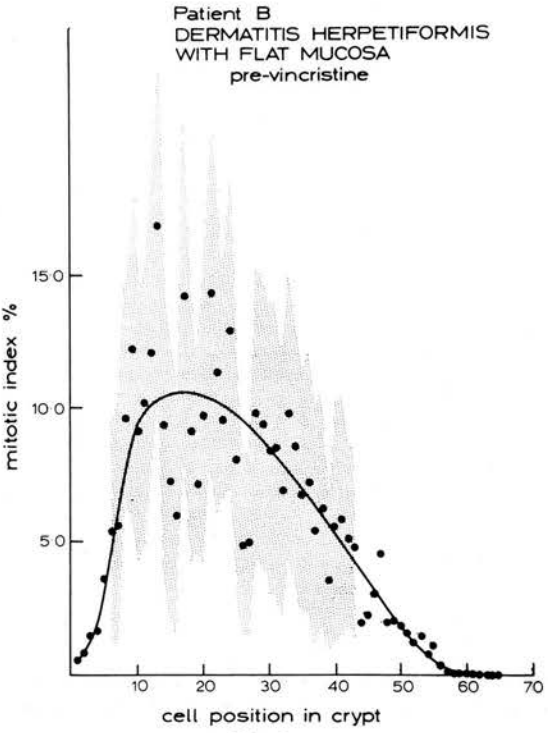
Patient B
DERMATITIS HERPETIFORMIS
WITH FLAT MUCOSA
pre-vincristine
mitotic index %
15·0
10·0
5·0
10
20
30
40
50
60
70
cell position in crypt

Fig. 3c

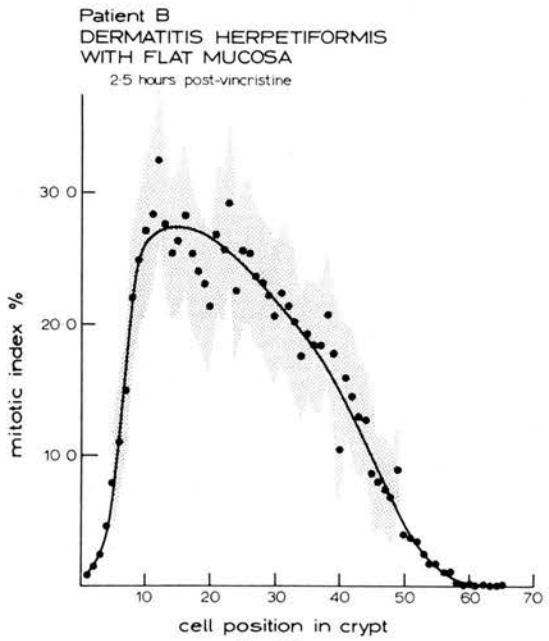

Fig. 3d

Values for the proliferative and maturation compartment sizes obtained from the pre-vincristine I_m distribution curves are given in Table 3. Despite a small decrease in I_p in patient B, because of the pronounced three dimensional increase in crypt size, there was an approximately two-fold increase in the number of proliferating cells per crypt. That is to say, that although there is a *relative* reduction in proliferative compartment size in the flat mucosa of patient B, an absolute increase in proliferative compartment size (in terms of cell number) has occurred because of the length (and girth) increase in the crypts of the flat mucosa. I_m values for the whole crypt column (obtained from pre-vincristine samples) are also given in Table 3. I_m was significantly higher ($p < 0.001$) in patient B, and this difference is of course accentuated when the I_m is calculated as a percentage of the proliferating population only. The values obtained for Tannock's constant were 0.71 for patient A and 0.72 for patient B.

The duration of mitosis, t_m, was calculated at each position (Wright *et al.*, 1972 b) and cumulative birth rate curves constructed; these are shown for patient A in Fig. 4a, and for patient B in Fig. 4b. The migration rate was 0.46 cell positions per h in patient A, and 1,65 in patient B (Table 4). Estimates of cell production rate show a four to five-fold increase in patient B. The transit times through the various compartments are given in Table 4, and it can be seen that despite the increased length of the crypt column in the flat mucosa of patient B, the transit time through the proliferative compartment is shorter. This is of course a reflection of the increased migration rate in patient B. The stem cell compartment was defined as those cells at the base of the crypt with transit times exceeding 20 h.

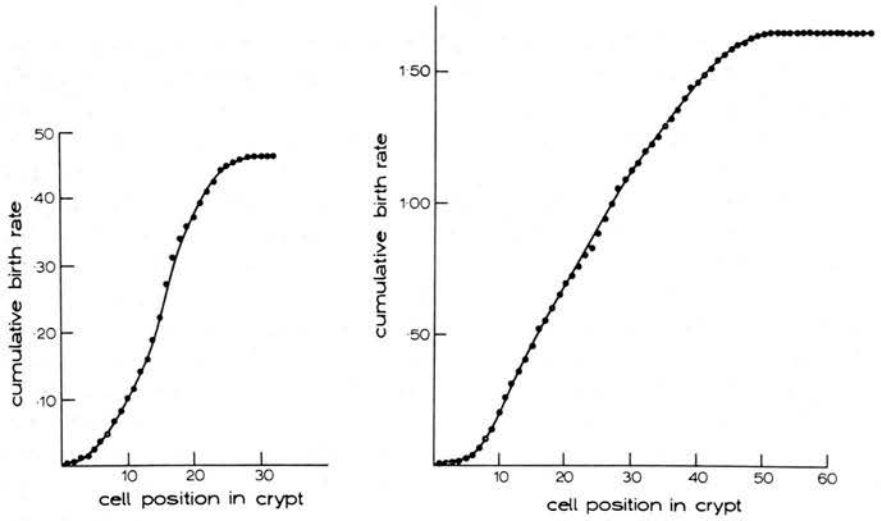

Fig. 4. Cumulative birth rate curves for patient A (4a), and patient B (4b). In each case the t_m values obtained from the vincristine study have been used in the calculation of the birth rate at each cell position

Table 4. Flux parameters in the human small bowel mucosa

	Migration rate (cell positions/h)	Crypt cell production rate (cells/crypt/h)	$T_{\text{prolif.}}$ (h)	$T_{\text{mat.}}$ (h)	$T_{\text{prolif.+mat.}}$ (h)
Patient A (villous mucosa)	0.46	10.2	116	15	132
Patient B (flat avillous mucosa)	1.65	50.2	60	15	75

This involved the lower 6 cell positions in each case. A similar principle was employed by Wright *et al.* (1972b) in their definition of rat stem cells.

Implicit in the above calculations are two assumptions concerning the mode of action of vincristine. Firstly that vincristine exerts its stathmokinetic (or metaphase arresting) action immediately upon injection, as is apparent in the rat studies; if vincristine does not block cells which had already embarked upon mitosis there would be a lag equal to the duration of mitosis before metaphase accumulation began, with consequent underestimation of the rate of entry into mitosis. The second assumption is that linear accumulation of metaphases occured. Both these assumptions have been confirmed using the multiple biopsy technique after vincristine injection (Wright, Watson, Morley, Appleton, Marks and Douglas, 1973).

Discussion

From the animal studies here described it is clear that the vincristine technique gives values for the crypt cell migration rate which compare well with ^{3}HTdr methods. Further, the estimate obtained for cell production rate (39 cells per crypt per h) is in agreement with that of Clarke (1971), and of Wright *et al.* (1974), who both used the crypt squash technique. One assumption implicit in the present method is that the crypt approximates to a cylinder, but the conformity of the cell production rates would appear to vindicate this assumption in this context.

An important point in the construction of the cumulative birth rate curves is the choice of t_m. Wright *et al.* (1972b) have explored the variation in migration rate when constant values for t_m are assumed to obtain at all cell positions; they concluded that accurate values for migration rate could only result when t_m is measured at each cell position and this variable t_m used in the calculation. The procedure is, of course more laborious than the crypt squash method, and can be criticised concerning the assumption made above; in its favour the method also gives information concerning the rate of migration of cells at all points within the crypt, a comparative estimate of the proliferating population, and estimates of transit time through the various compartments. Also, in our hands, reasonable values for crypt cell production rate have been obtained.

It should be noted that the value obtained for the migration rate using the movement of the 50% I_L peaks is slightly higher than that of Cairnie *et al.* (1965a); it may also be more accurate, as more crypts were enumerated in the present study, and the scatter of points about the line is reduced.

The problem of delineating the stem cell compartment at the base of the crypt is one which has been considered by several investigators (Cleaver, 1967; Lamerton, 1972) who have defined it on purely arbitrary grounds. Wright *et al.* (1972b) have studied the kinetics of these cells at the base of the crypt and showed that the bottom three position have a longer cell cycle time than that of cells in the proliferative compartment. It is possible that these longer cycling cells act as stem cells for the small bowel in the same way as haemopoietic stem cells (also of long cycle duration).

As in the animal studies, t_m calculated at each cell position was used in the construction of cumulative birth rate curves for the most accurate results in the human work; it is evident that there is a four-fold increase in migration rate, and, again assuming a tubular crypt structure in both control and flat mucosae, a four to five-fold increase in cell production rate in the flat mucosa of patient B. Although this assumption appears reasonable from observation of serial transverse crypt sections in each case, some accurate stereological model building would be required for complete justification.

The increased migration rate confirms the observations of Trier and Browning (1970) who, using an *in vitro* culture technique, involving labelling with tritiated thymidine, were able to demonstrate faster migration rates in biopsies from patients with the flat mucosa of gluten-sensitive enteropathy. On the other hand, there is nothing to support the conclusions of Creamer (1962) who suggested that crypt cells in flat mucosae moved upwards at a slower rate than normal crypt cells. The present results, together with those of a previous study (Wright *et al.*, 1973) indicate a hyperproductive proliferative state in flat mucosae.

It should be noted that the kinetic arguments presented all incorporate a tacit assumption of steady state conditions. The equations are readily altered to fit exponential conditions; steady state is a reasonable assumption in the normal mucosae (Schultze, Staack, Schmeer and Maurer, 1972), and while we have no prior indications of the growth conditions to be expected in flat mucosae, there is no reason to suppose that steady state conditions do not obtain.

As already mentioned, the use of mitotic index distribution curves to estimate the growth fraction would be expected to give higher values than those obtained from labelling index distribution curves (Wright *et al.*, 1973) and this is borne out by the observations of Cairnie *et al.* (1965 b) on the basis of the slow cut-off model. However, this usage can be expected to give a maximum value for the growth fraction and to demonstrate any major differences in proliferating population between two contrasted groups. An earlier experimental study (Wright *et al.*, 1972 b) showed that the differences between I_p measured from labelling index and from mitotic index distribution curves is not large; in the human studies the sizes of the proliferative compartments should therefore be regarded as maximum values.

In the human studies the delineation of the 'stem cell compartment' is difficult and needs some assumptions. The stem cell compartments in patients A and B have arbitrarily been defined on the basis of transit time; all cell positions with transit times exceeding 20 h being incorporated in the 'stem cell compartment'. This involves the lower 6 cell positions in both cases A and B. Further work is being undertaken to characterise this important portion of the crypt more accurately, considering Paneth cells and using techniques established experimentally in a previous study (Wright *et al.*, 1972 b).

Vincristine appears to be an excellent agent for studying small intestinal cell kinetics in animals and man. In each study there was no evidence that cells were passing the metaphase blockade, and because of the short arrest period employed, there was no apparent loss of metaphases due to degeneration (Aherne and Camplejohn, 1972). No evidence of deleterious side effects has been found in seven of our patients given the drug, and Camplejohn, Bone and Aherne (1973) have used the technique in 25 patients without mishap. Since *in vivo* techniques involving tritiated thymidine are potentially too dangerous for routine use, and crude mitotic indices are far from satisfactory, we suggest that the technique described is most suitable for the detailed investigation of small bowel proliferative status.

This work was supported by a grant from the North of England Branch of the Cancer Campaign. Skilled technical assistance was provided by Miss Doreen Hardy, Miss Elizabeth Robinson and Miss Glynis Stones.

Ethical Considerations. All patients received a full explanation of the procedure before informed consent was obtained. The project was presented to the Ethical Committee of the Newcastle University Hospital Group, and was approved under the following terms of reference.

1971. Dr. J. Marks. The use of vincristine with two Crosby capsule biopsies.

1972. Dr. A. Douglas. The use of vincristine in conjuction with the Quinton multiple biopsy machine.

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Cytokinetic studies of crypts in convoluted human small-intestinal mucosa

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Journal of Clinical Pathology, 1979, **32**, 462-470

Cytokinetic studies of crypts in convoluted human small-intestinal mucosa

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SUMMARY Forty-seven peroral biopsy specimens of duodenojejunal mucosa showing convolutions were obtained from patients with a variety of clinical disorders. These mucosal samples were divided into three groups according to the extent of the convolutions and the severity of the accompanying histopathological changes; the cytokinetic status of the crypts in the three groups was then analysed and compared. Those in which the mucosae were predominantly or totally convoluted (group 3) showed the most notable cytokinetic changes: crypts were hyperplastic and crypt cell production rate was markedly increased compared with the other two groups and with morphologically normal control mucosae.

In the case of one patient with mucosal changes of group 3 severity, additional studies were carried out using vincristine to produce metaphase arrest. The cell cycle time of 27 hours was greatly shortened compared with a control value of 45 hours.

We find that the presence of convolutions in small-intestinal mucosal biopsy specimens is accompanied by an increase in the rate of cell production from the crypts, which is presumably related directly or indirectly to the rate of loss of mature enterocytes from the surface of the mucosa. Furthermore, an increase in the proportion of convolutions may reflect an increase in the rates of cell production and cell loss.

In the group 3 convoluted mucosae the cytokinetic profile of the crypts resembled that of some of the flat avillous coeliac mucosae previously studied although the rates of cell production did not reach the levels attained by the most productive of the flat coeliac mucosae.

After the introduction of satisfactory methods for obtaining biopsy samples of small-intestinal mucosa by the peroral route it was soon observed that some jejunal mucosae presented a histological appearance intermediate between the normal villous state and the state of subtotal or total villous atrophy seen in untreated coeliac disease; this intermediate state was termed 'partial villous atrophy' (Doniach and Shiner, 1957). Subsequently it was found that a useful adjunct to histological examination is a preliminary examination of the biopsy specimen through a stereomicroscope (dissecting microscope) at low magnification under oblique illumination (Holmes *et al.*, 1961). It was suggested that the term 'convoluted mucosa' should be used to describe the stereomicroscopic counterpart of partial villous atrophy because of a resemblance to the surface of the cerebral hemispheres.

Unlike the finding of a flat avillous duodenojejunal mucosa, with relatively small numbers of crypt vestibules opening on to the surface of mosaic plateaux, which is so highly characteristic of untreated coeliac disease as to be virtually diagnostic in appropriate clinical circumstances, the finding of a convoluted appearance is of uncertain significance as an isolated observation, and all other aspects of the case have to be taken into account (Watson and Wright, 1974). However, occasional patients with untreated coeliac disease do show variation in mucosal appearances in multiple biopsy specimens, and in these cases a convoluted appearance is not uncommon. Furthermore, it is well established that convolutions develop as an intermediate stage in the morphological recovery of a flat mucosa after gluten withdrawal, or in the regression of a recovered

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Received for publication 8 November 1978

coeliac mucosa after re-exposure to gluten. In addition, convoluted duodenojejunal mucosae are quite commonly found in clinically mild or subclinical coeliac disease of dermatitis herpetiformis (Marks and Shuster, 1970) and in relatives of patients with coeliac disease (Robinson *et al.*, 1972).

It is not germane to the purpose of this paper to review the evidence relating to the incidence of a convoluted form of duodenojejunal mucosa in apparently healthy subjects in different parts of the world (Baker, 1973) or to the frequency of its association with such intestinal disorders as tropical sprue (Baker *et al.*, 1962; Swanson and Thomassen, 1965) or with various dermatological conditions (Marks and Shuster, 1970). In the literature we find that the term 'convoluted' has been used with different connotations by different workers and even by the same workers on different occasions; for example, it has been applied to mucosae with no more than single convolution (Shuster *et al.*, 1967), or to those mucosae only in which convolutions were predominant (Salem and Truelove, 1965; Marks and Shuster, 1970), or more restrictively to those mucosae which were wholly convoluted (Scott *et al.*, 1964). We have drawn a clear distinction between these categories of convoluted mucosae by allocating our biopsy specimens to one of three grades according to increasing severity of morphological abnormality based on well-defined stereomicroscopic and histopathological criteria; then by applying cytokinetic analytical techniques we have sought to establish whether there is a corresponding gradation of proliferative activity in the mucosal crypts. The findings have also been related to those which obtain in crypts of morphologically normal mucosae at one end of the spectrum and in the hyperplastic hyperproductive crypts of flat coeliac mucosae at the other. In one instance, viz, that of a patient with a completely convoluted duodenojejunal mucosa, additional cytokinetic data were obtained by comparing the findings in two mucosal biopsy specimens taken respectively before and at a suitable time interval after chemically induced stathmokinesis.

Material and methods

BIOPSIES

Forty-seven mucosal specimens were obtained by peroral biopsy of the duodenojejunal region. Most biopsies were taken in the middle of the day, which may minimise any diurnal variation of proliferative indices (Al-Dewachi *et al.*, 1976). The specimens were spread on glass and examined under the stereomicroscope; they were then fixed in neutral buffered 10% formol-saline and post-fixed in formol-corrosive. The tissues were processed in the usual manner and embedded on edge in paraffin wax; serial sections were prepared at a thickness of 3 μm and stained with Harris's haematoxylin.

CLASSIFICATION OF MORPHOLOGICAL APPEARANCES

The biopsy specimens were subdivided into three groups (1, 2, and 3) on the basis of increasing severity of morphological abnormality upon stereomicroscopic and histological observation; the criteria are set out in Table 1. Some specimens showed only one or two convolutions, distributed among ridges, leaves, and fingers in varying proportions, while others were completely or almost completely convoluted; the remainder fell between these two extremes. The presence of linear and curvilinear ridges did not contribute to the classification, since the aim of this study was to ascertain the significance of convolutions. The histological appearances ranged from normal to a state of undoubted abnormality, with crypt hyperplasia and incomplete villous atrophy approaching that seen in untreated coeliac disease. The biopsy specimen was placed in its appropriate group on the basis of whether stereomicroscopic or histological grading was worse, in the event that the two differed. The clinical diagnoses in each group are given in Table 2.

MORPHOMETRIC VARIABLES

Counting techniques were the same as those previously described (Wright *et al.*, 1973a). In each

Table 1 *Criteria for allocating convoluted mucosae to appropriate group according to increasing severity of morphological abnormality*

	Group 1	*Group 2*	*Group 3*
Stereomicroscopic	One or at most a few convolutions distributed among ridges, leaves, and fingers in various proportions	A range of appearances falling between the extremes of groups 1 and 3	Completely or predominantly convoluted
Histological	No notable abnormality	Evident shortening of villi; crypt hyperplasia slight or inapparent; mildly increased numbers of inflammatory cells in lamina propria; surface epithelium normal	Pronounced villous shortening (less than subtotal); obvious crypt hyperplasia; readily apparent increase in density of inflammatory cell infiltration of lamina propria; abnormality of surface epithelium (not mandatory)

Table 2 *Clinical diagnosis related to morphological groups*

Group 1		*Group 2*		*Group 3*	
Dermatitis herpetiformis (DH)	5	DH	3	DH	7
Relatives of DH patients	2	Coeliac disease	3	Coeliac disease	2
Anaemia (cause undetermined)	1	Eczema	2	Diarrhoea (colonic carcinoma)	1
Coeliac disease (treated)	1	Failure to thrive	2	Giardiasis	1
Diarrhoea (cause undetermined)	1	Crohn's disease (?)	1	Jejunitis (?cause)	1
Eczema	1	Giardiasis	1	Malabsorption (Hodgkin's disease)	1
Failure to thrive (child)	1	Iron-deficiency anaemia	1	Psoriasis	1
Giardiasis	1	Malabsorption (?cause)	1		
Giardiasis (post-treatment)	1	Malnutrition	1		
Psoriasis	1	Postvagotomy diarrhoea	1		
		Short stature	1		
		Thymic alymphoplasia	1		
Totals	15		18		14

case at least 30 crypts were selected in which the base, middle, and mouth of the crypt were all in the plane of section. Care was taken to ensure that each crypt was analysed only once, and that the source of the sections was unknown to the observer. In each crypt the 'left-hand' column of cells was numbered, counting from the bottom upwards to the cryptvillus junction, and the cell positions of the mitotic nuclei were recorded. The number of cells present in a column (the crypt column length) and the number of crypt columns (the column count) were recorded. The latter was measured by turning the block and cutting sections tangentially to the bowel.

The data were analysed as previously described; results were expressed on a crypt whose length was the mean crypt column length for the group, and the mitotic index was then plotted against cell position in the crypt to give a *mitotic index distribution curve* for each group (Wright *et al.*, 1973a).

STATHMOKINETIC STUDY

The patient studied was a man (RM) aged 25 with a clinical diagnosis of dermatitis herpetiformis and coeliac disease, who at the time of the present investigations showed no clinical or biochemical evidence of malabsorption. On day 1 a biopsy capsule was passed, and a mucosal biopsy of the duodenojejunal region was obtained in the normal way at 1330 hours. On day 2, the capsule was passed to the same position as on the previous day, and at 1100 hours vincristine sulphate (Oncovin, Eli Lilly) was rapidly infused intravenously in a dose of 0·045 mg/kg body weight. At 1330 hours the capsule was fired and a second mucosal biopsy was obtained. Care was taken to ensure that no appreciable movement of the capsule occurred during the stathmokinetic period following vincristine injection. The method of tissue processing, sectioning, and analysis was as before. Only prophases and metaphases were seen in the post-vincristine sample, indicating that the blocking effect of vincristine was still operative when this sample was taken.

In the pre-vincristine biopsy 204 crypts were suitable for analysis, and in the post-vincristine sample 210. The column count was estimated from transverse sections of 100 crypts from the pre-vincristine sample.

The correction factor of Tannock (1967) was measured, using a calibrated eyepiece graticule, in 139 transverse sections containing metaphases from the post-vincristine biopsy specimen.

Results

MORPHOLOGICAL GROUPS

Using the criteria of Table 1, 15 specimens were assigned to group 1, 18 to group 2, and 14 to group 3. There was good correlation between stereomicroscopic and histological appearances, although this was not perfect.

MORPHOMETRIC VARIABLES

These are given for the three groups in Table 3 where they are compared with results obtained in a series of 75 biopsies in which the stereomicroscopic and histological appearances were considered to be within normal limits (Wright *et al.*, 1973a). There was enough material for the column count to be determined from 100 crypt cross sections in each of seven specimens from group 1, eight specimens from group 2, and six specimens from group 3. It is evident that there are increases in both crypt column length and column count with increasing severity of morphological changes. In all groups the crypt length and the column count were significantly greater than control values. Wimber and Lamerton (1963) reported that the product of the crypt length and column count gave a value for the crypt population which compared reasonably well with results obtained from counts of squashed, microdissected crypts. On this

Table 3 *Kinetic and morphometric measurements in convoluted groups compared with controls and adult coeliacs*

	Crypt column length (cells)‡	*Column count (cells)*‡	*Crypt population (cells)*	*Mitotic index (%) (corrected)*	*Growth fraction*	*Proliferating population (cells)*	*Migration rate (cell positions/h)*	*Crypt cell production rate (cells/crypt/h)*
Controls (adult)*	31·9 ± 0·1	24·5 ± 0·2	780	2·1	0·72	560	0·7	17
Group 1 convoluted	37·1 ± 0·3	25·5 ± 0·2	950	2·4	0·74	700	0·9	23
Group 2 convoluted	39·0 ± 0·3	27·1 ± 0·2	1060	2·6	0·69	730	1·0	28
Group 3 convoluted	48·9 ± 0·6	30·8 ± 0·3	1510	3·8	0·77	1160	1·9	58
Coeliac disease (adult)† flat mucosa	74·1 ± 0·6	41·2 ± 0·3	3050	3·6	0·55	1370	2·6	107

*A series of 75 biopsies of morphologically normal mucosa (see Wright *et al.*, 1973a).
†A group of 13 biopsies of flat, avillous mucosa of adult coeliac disease (see Wright *et al.*, 1973a).
‡±SE.

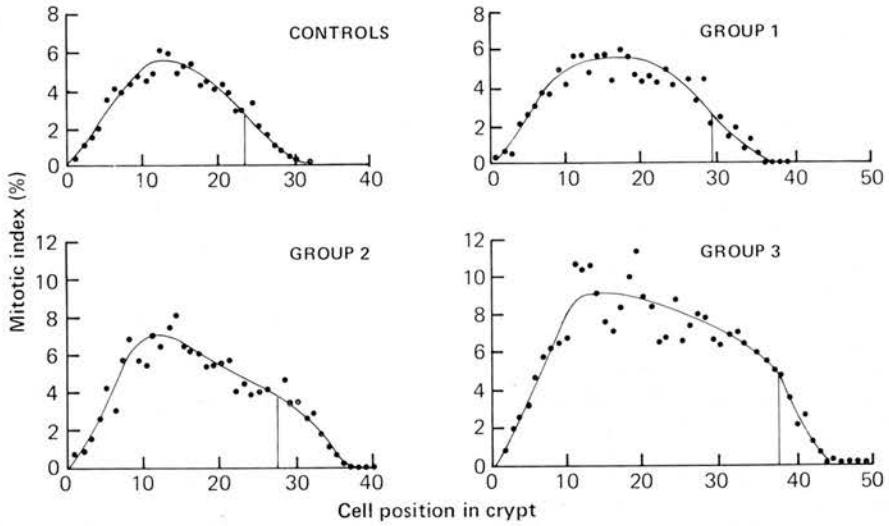

Fig. 1 *Mitotic index distribution curves for convoluted groups 1, 2, and 3 compared with a mitotic index curve for a group of 75 adult control patients with morphologically normal jejunal mucosae.*

basis the results indicate increases in crypt population with increasing severity of morphological abnormality, and group 3 shows a doubling of crypt population compared with controls.

MITOTIC INDICES

The mitotic indices, with control values, are shown in Table 3. Although groups 1 and 2 show small increases compared with normal, only group 3 shows a significant increase in mitotic index.

MITOTIC INDEX DISTRIBUTION CURVES

Figure 1 shows the variation in mitotic index, plotted as a function of cell position in the crypt, for groups 1, 2, and 3, and, for comparison, the mitotic index distribution curve for the group of adult controls is also shown. The curves are similar: low mitotic indices are seen in basal cell positions, and then the mitotic index rises to a maximum in the proliferative compartment of the crypt. In group 3, the maximum index attained in the proliferative compartment exceeds 6% compared with a peak index of less than 4% in group 1 and controls. If all cells within the proliferative compartment are in the cell cycle, then the portion of the crypt delineated by the 50% peak point will be equivalent to the growth fraction (Cleaver, 1967). Even if we do not make this assumption the 50% peak point still gives a comparative measurement of the cut-off position in the crypt, or the point at which cells cease proliferating and enter the maturation compartment.

All groups show growth fractions which are close

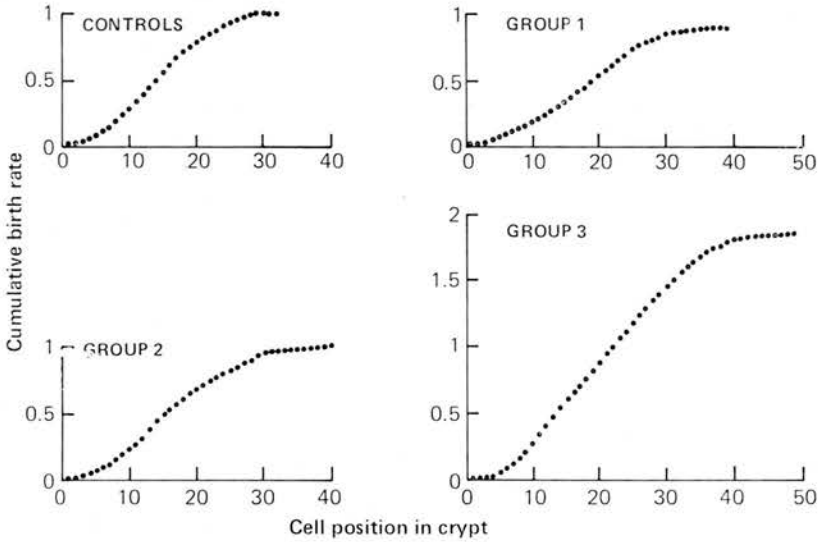

Fig. 2 *Cumulative birth rate curves for convoluted groups 1, 2, and 3 compared with adult controls (units for cumulative birth rate are cells/cell position/h).*

to control values. However, if we calculate the total number of proliferating cells per crypt from the growth fraction and the crypt population, then group 3 contains more than double the normal complement of proliferating cells (Table 3); much smaller increases are shown for groups 1 and 2.

It can be seen from Table 3 that the total number of proliferating cells is close to that previously found in untreated adult coeliac disease (Wright *et al.*, 1973a), in spite of the fact that the total crypt population is only half of that in coeliac disease.

FLUX PARAMETERS

The migration rate, or rate of migration of crypt cells in cell positions transvered per hour, and the crypt cell production rate (in cells/crypt/h) can be calculated from the mitotic index distribution data; the birth rate at each cell position was calculated as previously described (Wright *et al.*, 1972), assuming that the duration of mitosis is one hour at each cell position (Wright *et al.*, 1973b) in all cases. The efflux (in cells/cell/h) at the top of the crypt column (or at any cell position) is equal to the sum of the birth rates in all preceding cell positions; if crypt cells migrate because of mitotic activity in lower cell positions, the efflux at any cell position is also equal to the migration rate.

Cumulative birth rate curves for the three convoluted groups are shown in Figure 2. Group 3 has a larger maximum migration rate than groups 1 and 2, attaining a rate of 1·9 cells per hour at the top of the crypt. The migration rates in groups 1 and 2 are close to the value found in controls (see Table 3).

From the cell production rate per crypt column and the column count (Table 3) we can estimate the crypt cell production rate. Table 3 shows that group 3 has a crypt cell production rate double that in the other two groups and more than three times that in control mucosae. However, values for group 3 are not as large as those found in the completely avillous flat mucosa, which is more characteristic of untreated coeliac disease (Wright *et al.*, 1973a).

STATHMOKINETIC STUDY

Both biopsies from the patient studied showed a completely convoluted mucosal surface on stereomicroscopy, while histological examination showed group 3 type changes. Morphometric measurements for this patient are shown in Table 4. Crypt column length and column count are close to the means for group 3 (Table 3), and consequently the estimate of total crypt population is similar.

In the period of metaphase arrest, which was 2·5 hours, the mitotic index for the crypt rose (allowing for Tannock's correction factor, which was 0·69) from 3·2% to 9·6%, a rise of 6·4% in 2·5 hours. Since metaphase arrest was complete, the rate of entry into mitosis was 2·6% of crypt cells per hour, assuming that vincristine took immediate effect.

An estimate, albeit an imprecise one, of the

ble 4 *Morphometric and kinetic measurements in a patient with convoluted mucosa of dermatitis herpetiformis compared th patients with morphologically normal and with flat avillous mucosae*

	Crypt column length‡ (cells)	*Column count (cells)‡*	*Crypt population (cells)*	*Growth fraction*	*Proliferating population (cells)*	*Migration rate (cell positions/h)*	*Crypt cell production rate (cells/crypt/h)*	*Mitotic duration (h)*	*Apparent cell cycle time (h)*	*Cell cycle time (h)*	*Rate of entry into mitosis (% per hour)*
nvoluted mucosa	49·9 ± 0·2	29·3 ± 0·7	1460	0·72	1050	1·7	50	1·2	27	19	2·6
ult control*	31·9 ± 0·2	22·3 ± 0·6	710	0·81	580	0·5	10	1·1	45	36	1·5
t mucosa†	67·0 ± 0·5	30·4 ± 0·5	2040	0·60	1220	1·7	50	1·3	26	16	2·7

patient with morphologically normal mucosa studied by the two biopsy vincristine method (Wright *et al.*, 1975).
patient with the flat, avillous mucosa of dermatitis herpetiformis studied with the two biopsy vincristine method (Wright *et al.*, 1975).
e data for both of these patients have been re-analysed so as to give results strictly comparable with those for the patient with the convoluted mucosa.
s involved using Tannock's correction factor and the assumption of an exponential age distribution. Figures given here are therefore more accurate than he reference cited.
SE.

duration of mitosis (t_m) can be calculated from the equation

$$t_m = \frac{I_m(0) \cdot t}{(I_m(t) - I_m(0))} \quad (1)$$

where $I_m(0)$ is the mitotic index at time zero, t is the duration of metaphase arrest (2·5 hours), and $I_m(t)$ is the mitotic index at time t. This gives a value of 1·2 hours, which is similar to control values (see Table 4).

The apparent cell cycle time, $T_{c(a)}$, which is the cell cycle time assuming that all crypt cells are proliferative, can be calculated, assuming an exponential age distribution, from

$$T_{c(a)} = \frac{t \ln 2}{(I_m(t) - I_m(0))} \quad (2)$$

This gives a value of 27 hours, which is significantly different from control values (Table 4) and is close to values found in the flat mucosa of coeliac disease. To calculate the actual cell cycle time we must multiply $T_{c(a)}$ by the growth fraction. A value of 19 hours is obtained.

The mitotic index distribution curves before and after vincristine are shown in Figure 3. In the pre-vincristine sample there is considerable variation in individual mitotic indices, but in the post-vincristine sample, because of the higher mitotic indices, variation is relatively less. Table 4 shows that the growth fraction and the proliferating population are similar to those found in group 3. Assuming that there is no movement of the arrested metaphases relative to the interphase crypt cell column, the rate of entry into mitosis at each cell position in the crypt can be calculated; this is shown in Figure 4. Data are available for cell positions 3 and above: low values are apparent at the low cell positions, but from cell positions 10 to 40, values are about 0·4 cells/cell/h.

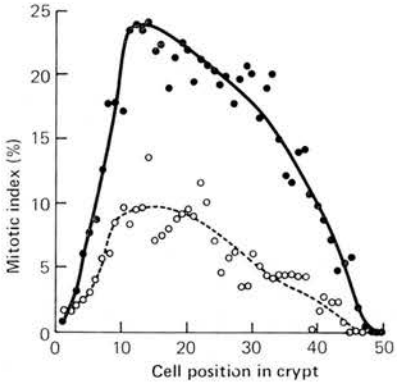

Fig. 3 *Mitotic index distribution curves in the pre-vincristine and post-vincristine biopsy specimens for patient RM (group 3 convoluted mucosa):* ○ *pre-vincristine;* ● *post-vincristine.*

Finally, as cells decycle at the top of the crypt, mitotic rates decline.

From equation 1 the duration of mitosis can be calculated for each cell position, and hence the birth rate at each cell position can be calculated. The resulting cumulative birth rate curve is shown in Figure 5. The maximum migration rate attained is 1·7 cell positions per hour, and the crypt cell production rate is 50 cells/crypt/h.

Discussion

The results obtained here allow us to draw several conclusions about the physiological significance of the presence of convolutions in a jejunal biopsy, and also about the relationship between convoluted and flat jejunal mucosae.

The changes in group 3 are certainly most striking; this group showed an increase in total crypt cell

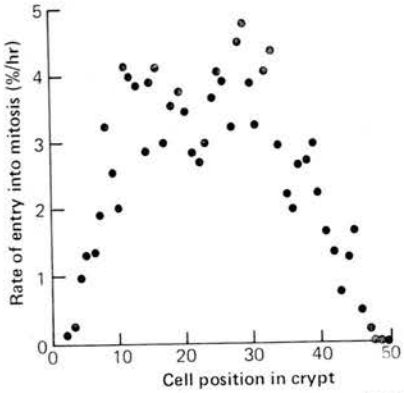

Fig. 4 *Variation in rate of entry into mitosis (%/h) with cell position in crypt of patient RM.*

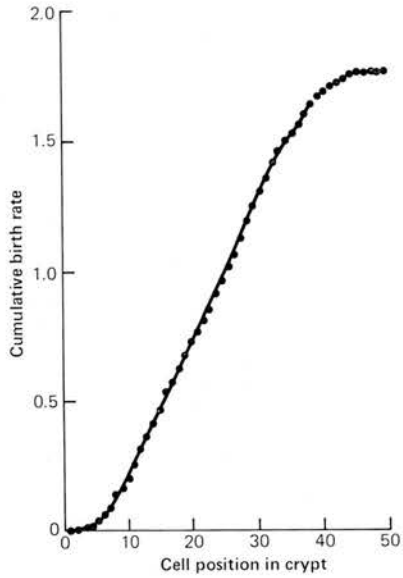

Fig. 5 *Cumulative birth rate curve for patient RM (units for cumulative birth rate are cells/cell position/h).*

population, an increased number of proliferating crypt cells, and an elevated crypt cell production rate. Furthermore, analysis of a metaphase arrest study on a patient with the mucosal appearances of group 3 showed a significant decrease in apparent cell cycle time compared with control mucosae. It is concluded that mucosae with the appearances of group 3, that is, predominantly or completely convoluted with pronounced villous shortening and with crypt hyperplasia, are in a hyperproductive state. However, this increase in crypt cell production is not as large as that found in flat mucosae (Wright *et al.*, 1973a and b), although there may be some individual overlap in kinetic terms; for example, the migration rate and crypt cell production rate in the case for which we have these data were close to those found in a patient also with dermatitis herpetiformis but with a flat mucosa (Table 4).

An increase in crypt cell production rate presumably occurs in response to an increased rate of surface cell loss. Since in our group 3 the cell production rate is 58 cells/crypt/h, compared with 107 cells in flat mucosae (see Table 3), we may infer, by assuming that a state of balance is attained, that cell loss rate is not as large in convoluted mucosae as in flat mucosae. If we postulate that stable states of altered mucosal morphology in the small intestine are a reflection of the balance between cell production and cell loss, then our data are consistent with the hypothesis that a convoluted state represents the adjustment to a moderate increase in rate of cell loss, while a flat, avillous state is the result of a severe increase.

There appear to be at least three different mechanisms by which the intestinal crypt might increase its cell production rate to compensate for an increased rate of cell loss. Firstly, an increase in the crypt size, with an absolute increase in the population of proliferating cells, will lead to an increase in cell production rate although the growth fraction may be unaltered or even decreased; this response is evident in all of the convoluted groups. Secondly, an increase in the size of the proliferative compartment at the expense of the maturation compartment would increase the growth fraction and so lead to an increase in proliferation, but this response has not been observed in this study. Thirdly, a decrease in cell-cycle time would also increase the cell production rate; this has been shown to occur in the one patient with mucosal changes of group 3 severity so studied, but we have no information as to the occurrence of such a change in the other mucosae in this group or in those of groups 1 and 2. Consequently, we can only speculate as to the possible interrelationship of these compensatory responses. It seems likely that they are induced by some critical level or levels of cell-loss rate but they could be activated simultaneously or sequentially, and in the latter event they might or might not overlap. Observations of flat avillous mucosae in coeliac disease during morphological recovery after gluten withdrawal suggest that the convoluted state is intermediate between normality and total villous atrophy. Conversely, gluten challenge of a coeliac patient, whose flat mucosa has reverted to normality on a gluten-free diet, leads to a transient convoluted state during relapse to total villous atrophy. Our morphometric and cytokinetic findings are fully consistent with this inference

although the same conclusion could not be established from our findings *per se*.

The morphological status, morphometric variables, and cell-kinetic characteristics of the specimen from the patient on whom stathmokinetic studies were performed place it in group 3 convoluted. The small differences in migration rate and crypt-cell production-rate from the means of that group may be explained in terms of the approximations to the mitotic durations that were used in the calculations.

In the analysis of the vincristine study we have made several assumptions: firstly, since only two biopsy specimens were available, that vincristine arrests metaphases immediately (Wright *et al.*, 1973b); secondly, that the age distribution of cells is exponential (Cairnie *et al.*, 1965; Appleton *et al.*, 1977); and, thirdly, that the distribution of mitoses in the two biopsy specimens remained the same. Inspection of the mitotic index distribution curves before and after vincristine (Fig. 3) shows that the 50% peak point has moved from cell position 35 in the pre-vincristine sample to cell position 37 in the post-vincristine sample; therefore the distributions are reasonably similar.

We have demonstrated that there is a correlation between increasing prevalence of mucosal convolutions and a stepping-up of proliferative activity within the mucosal crypts, although the basis of this correlation remains a matter for speculation. It is probable that the formation of convolutions represents a morphological accommodation to a reduced population of surface cells (enterocytes), but this will be confirmed only when reliable methods have been developed for enumerating the surface cells on these complex structures. We suggest that our findings constitute additional evidence for the view that a convoluted duodenojejunal mucosa is not a definitive or end-stage pathological change but that it represents an intermediate stage between normality and a flat mucosa with total villous atrophy. In conclusion, we recommend that the term 'convoluted' as applied to small-intestinal mucosa be restricted, unless otherwise qualified, to connote degrees of abnormality which we have used to define our group 3. The term 'partial villous atrophy' should be discarded (Watson and Wright, 1974), and, despite the deprecatory attitude of some authorities (Perera *et al.*, 1975), we remain convinced that stereomicroscopy is a useful adjunct to histological assessment.

This work was supported by a grant from the North of England Cancer Research Campaign.

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Cell Proliferation in the Gastrointestinal Tract

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PITMAN MEDICAL

6.3 Cell Proliferation in Normal, Convoluted and Avillous Small-intestinal Mucosae of Man

A J Watson, N A Wright and D R Appleton

6.3.1 INTRODUCTION

Coeliac disease is a condition in which susceptible individuals develop a malabsorption syndrome, with characteristic morphological and functional abnormalities of the small-intestinal mucosa, when their diet contains items based on the grain of wheat, rye, barley and probably oats (32). The starch fraction is entirely harmless and it is the protein fraction gluten which produces the deleterious effects. Of the two proteins which make up gluten, the alcohol-soluble gliadin (168) is the harmful agent and the property is still present in a water-soluble peptide fraction following peptic-tryptic digestion of wheat gliadin (238). However, precise definition of the responsible factor at molecular level has not as yet been achieved despite intensive efforts over a period of some two decades. The assumption that the small-intestinal mucosa in coeliac patients might lack an enzyme which would normally break down the harmful digestion product of gliadin was widely accepted at one time and cannot yet be entirely rejected on experimental evidence (310). An additional, or alternative, pathogenetic mechanism for the mucosal changes may be an abnormal immunological response involving an antigen-antibody reaction or a form of cell-mediated immune reaction (682).

There is good evidence that susceptibility to coeliac disease is genetically determined (444). Affected individuals are likely to be of northwestern-European, especially Irish, origin and there is a particularly high prevalence of the condition in the West of Ireland (492). Among first-degree relatives of patients coeliac disease is much more common than in the population at large and, even if asymptomatic, many relatives show coeliac-type mucosal abnormalities (588). There is a strong connection with the histocompatibility antigens HLA-B8 and HLA-DW3 which show linkage disequilibrium and may be linked to a separate coeliac-disease gene (682).

Other names used for coeliac disease include coeliac sprue, non-tropical

sprue, idiopathic steatorrhoea and gluten-induced enteropathy. Like many other conditions defined initially in terms of signs and symptoms (syndromes) precise identification of coeliac disease as a specific nosological entity had to await discovery of the aetiological role of gluten. Towards the end of the Second World War the population of the Western Netherlands experienced a period of severe privation with shortages of foodstuffs including those based on wheat or rye. As adults grew lean, coeliac children thrived, and the incidence of the disease fell until conditions were restored to normal following the end of the war. The significance of these occurrences was elucidated by the Dutch paediatrician W K Dicke (167). With the subsequent introduction of the peroral biopsy technique (139, 232, 597, 644) the histopathology of the small-intestinal mucosa in untreated coeliac disease has been reported in great detail (1, 178, 600, 768), together with the sequence of changes by which normal morphology is restored during successful treatment, and the mode of reversion when treatment is discontinued (628). Valuable insights into the nature of the changes have also been provided by the use of additional techniques such as enzyme histochemistry (576), transmission electron micros copy (601, 645), and scanning electron microscopy (31, 454).

We can now define coeliac disease as a condition resulting from a probably permanent state of intolerance to dietary gluten associated with a flat avillous duodeno-jejunal mucosa on stereomicroscopy, usually with a mosaic pattern, and with characteristic histological changes, namely total villous atrophy, crypt hyperplasia, and a chronic inflammatory reaction. Strict adherence to a gluten-free diet leads to clinical and morphological improvement, but there is a liability to relapse following reintroduction of gluten.

The so-called animal models of coeliac disease are no more than morphologi cal analogues of crypt-hyperplastic, villus-atrophic mucosae (271). Despite the limitations imposed by ethical and technical considerations there is no substitute for the direct study of the human disorder. Much can be learned about the proliferative status of normal and abnormal small-intestinal mucosae from the analysis of routine diagnostic biopsies, and the reasons for the increased numbers of mitotic cells in the crypts of coeliac mucosa can be investigated using stathmokinetic techniques in a small group of volunteers. We have already reported separately some of the results of these studies (768, 769, 814, 817, 820–823).

6.3.2 MATERIALS AND METHODS

6.3.2.1 BIOPSIES USED FOR DIAGNOSIS AND ANALYSIS OF MITOTIC INDICES

The biopsy specimens were obtained perorally from the vicinity of the duodeno-jejunal junction by means of a modified Crosby capsule; they were

taken as near midday as possible to minimise the effect of circadian variation, retrieved without delay, and observed under the stereomicroscope at low magnification while being gently spread out for transfer, mucosal surface upwards, onto a small piece of glass. Primary fixation was in 10 percent neutral buffered formol-saline, and detailed inspection of the mucosal surface was best carried out after 24 h fixation when obscuring mucus and debris could safely be removed by gentle brushing. Post-fixation in a 5 percent solution of mercuric chloride in formol-saline was carried out to improve the quality of fixation and to give better nuclear staining, and the specimen was processed through to paraffin wax and embedded on edge. Some 200 serial sections 3 μm thick were cut, most of which were stained by Harris's haematoxylin and used for analysis of the crypts. For diagnostic assessment several sections were stained by haematoxylin and eosin and by the periodic-acid Schiff method after amylase digestion; flat coeliac mucosae were also routinely stained by the haematoxylin-van Gieson and by the picro-Mallory methods to detect collagenisation of the lamina propria.

6.3.2.2 CLASSIFICATION OF BIOPSIES

The biopsy specimens studied were assigned to one of 3 main categories on the basis of their morphological appearances as seen on stereomicroscopic and histological examination. Although the value of stereomicroscopy has been disparaged (520, 599) both forms of examination are essential if errors are to be avoided. If stereomicroscopy has been neglected, the paraffin-embedded specimen can be processed back to 70 percent spirit without sustaining any harm.

Normal morphology. These specimens showed a normal villous appearance on stereomicroscopy and conformed to the conventional appearance of normality on histological examination. It is relatively unusual to find a specimen from the duodenojejunal region showing exclusively a population of tall finger-shaped villi, and narrow or broad leaf-shaped forms were accepted as normal; the presence of curved or angular leaves, of joined leaves, of straight ridges or sinuous ridges among the more regular leafy villi did not debar specimens from inclusion in this category. Specimens showing convolutions were invariably excluded.

Convoluted mucosae. The stereomicroscopic appearance of a small-intestinal biopsy is described as convoluted if individual villi have been wholly or largely replaced by a complex of intertwining tortuous ridge-like structures, so that there is a certain resemblance to the surface of the cerebral hemispheres (Figure 6.3.1). The height of the convolutions often appears comparable to that of normal villi, but frankly atrophic forms are sometimes encountered. The inexperienced observer may overlook isolated or scattered convolutions, but is

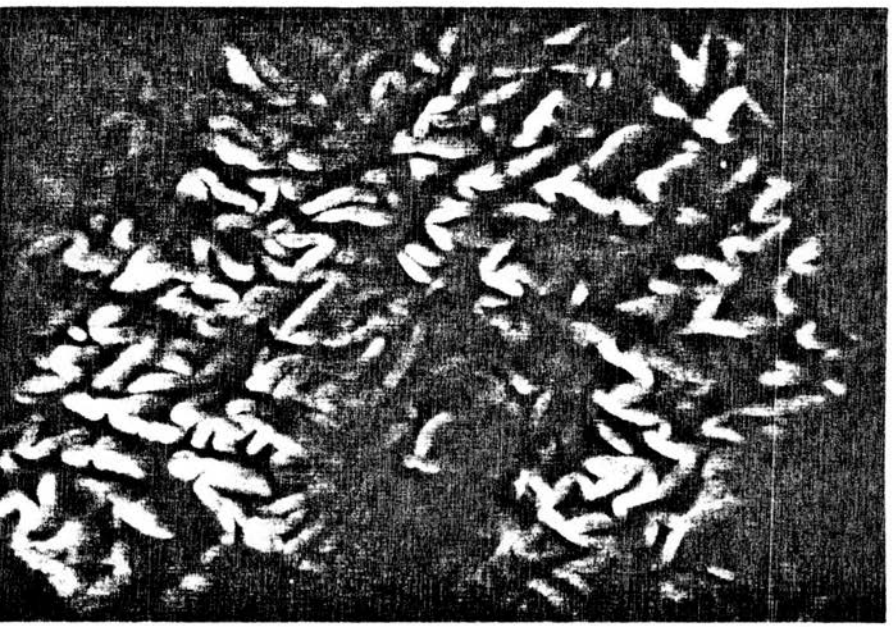

Figure 6.3.1 A peroral biopsy of proximal small-intestinal mucosa showing replacement of villi by so-called convolutions; this specimen would be assigned to group 3 (×16)

Table 6.3.1 Combinations of changes which characterise convoluted mucosae assigned to each of the three arbitrarily defined groups

Mucosal group	Stereomicroscopy	Histology
3	Surface completely or predominantly (over 70%) convoluted	Villi short and stubby; crypts obviously lengthened; surface epithelium abnormal; obvious increase in density of inflammatory cell infiltration
2	From 10 to 70% of surface occupied by convolutions	Some shortening of villi apparent on inspection; lengthening of crypts slight or inapparent; surface epithelium normal; density of inflammatory cell infiltration normal or just perceptibly increased
1	Not more than a few convolutions, distributed amongst leaves and ridges, and occupying less than 10% of surface	Normal or nearly so

more likely to misinterpret other angular, or sinuous formations as convolutions. The histological appearances of such specimens range from virtual normality to a state of severe villous atrophy with crypt hyperplasia and other changes approaching in severity those of untreated coeliac disease. For the purpose of analysis we have subdivided the convoluted mucosae into 3 groups on the basis of increasing severity of morphological abnormality according to the criteria set out in Table 6.3.1. In the event of a disparity between the severity of the stereomicroscopic and the histological changes, the more severe of the two assessments determined the group to which the specimen was assigned.

Flat avillous coeliac-type mucosae. The characteristic stereomicroscopic and histological appearances of the specimens assigned to this category are illustrated in Figures 6.3.2 and 6.3.3 and are so well established that further description is unnecessary.

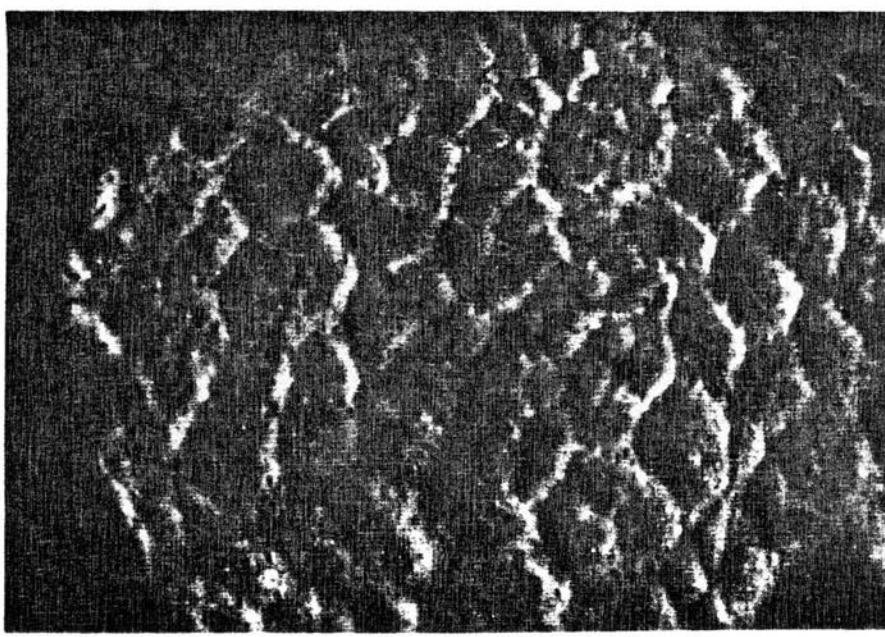

Figure 6.3.2 A peroral biopsy of proximal small-intestinal mucosa from a patient with untreated coeliac disease. This is a flat avillous mucosa showing the characteristic mosaic pattern. Some of the hyperplastic crypts open directly on to the surface, but it is more usual for several crypts to open into a short vestibular compartment just below the mucosal surface. The vestibular orifices are readily apparent on the surface of the mosaics (×16)

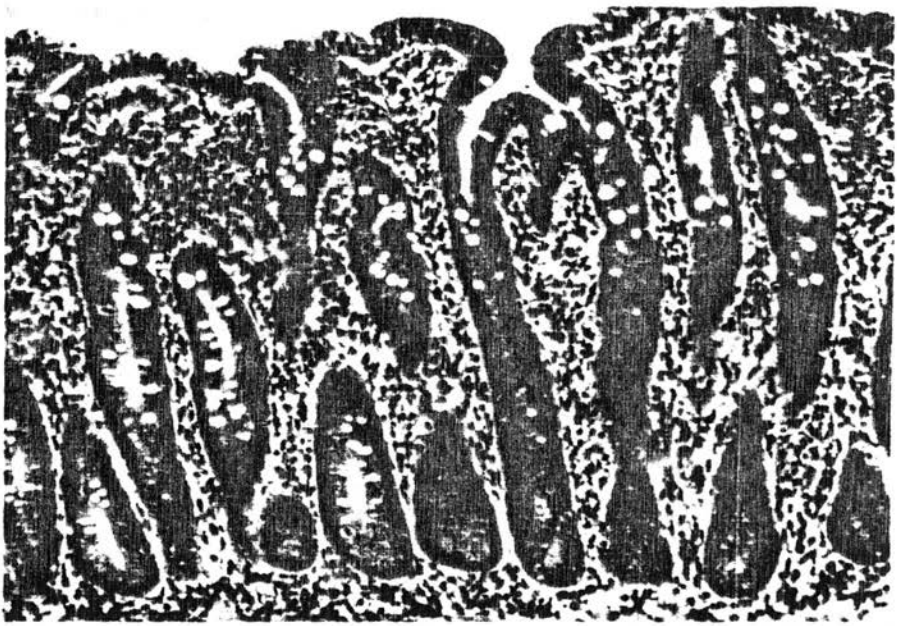

Figure 6.3.3 This shows the typical histological findings in the flat avillous mucosa from an untreated coeliac patient. There is total villous atrophy, but the crypts are hyperplastic and greatly elongated; dividing epithelial cells are plentiful and occur to a high level in the crypts; two crypts can be seen opening into a shared vestibule; the surface epithelium shows pseudostratification of nuclei and numerous intraepithelial lymphocytes; the lamina propria is heavily infiltrated by plasma cells, small and large mononuclear cells and eosinophils. (Haematoxylin and eosin ×150)

6.3.2.3 PATIENTS

The majority of patients had had a single biopsy sample of the proximal small-intestinal mucosa taken by the peroral route for diagnostic purposes. A large proportion of the samples were taken to exclude or confirm the diagnosis of coeliac disease. Many were taken from patients, with psoriasis or other dermatoses, who were possibly suffering from dermatogenic enteropathy (452, 650), and these investigations led to the discovery of the association between dermatitis herpetiformis (DH) and coeliac disease, the latter usually being subclinical (453, 651).

Our control patients, defined in terms of their having a morphologically normal biopsy, comprised 75 adults and 10 children less than 8 years old. There

were 62 patients with flat coeliac-type mucosae; 13 adults and 29 children with untreated coeliac disease and 20 adults with DH. The 47 patients with convoluted mucosae constituted a heterogeneous group in terms of clinical diagnosis, though it is notable that there were 15 with DH and 2 more who were near relatives of DH patients. There were also 6 coeliac patients in various stages of remission or relapse, and 4 patients with giardiasis.

6.3.2.4 METHODS OF ANALYSIS

In all subjects the objective was to analyse 30 crypts, each of which had been axially sectioned so as to include the base, middle and mouth of the crypt in the plane of section. The 'left-hand' column of cells was enumerated from the bottom to the mouth of the crypt as seen in section. In villous mucosae this corresponds approximately to the junction of crypt and villus, whereas in avillous coeliac mucosae the mouth is represented by the apparent junction of the crypt with the surface epithelium; it is likely in some instances to include part of the crypt vestibule. The number of cells in this column will be referred to as the crypt length and provides a measure unaffected by tissue shrinkage resulting from fixation and processing. Within the crypt column the positions of all mitotic cells were recorded and the distribution of mitotic index (I_m) relative to a crypt of length equal to the mean of the appropriate group was determined by pooling data for all crypts examined in that group (82, 814).

We have explained elsewhere (28) how a useful description of the extent of proliferation up the crypt, the cut-off position (83, 84, 668), can be found from stathmokinetic experiments, provided an estimate of the cell cycle time is available. We are unable to apply this method to our human material because FLM studies cannot be undertaken, and instead have derived from the mitotic index distributions estimates of the cell position at which I_m falls to 50 percent of its maximum value (120); we term this the half-maximum position, HMP. If all cells below this position were proliferating with the same cell cycle time, the cut-off position would be half this distance from the bottom of the crypt.

6.3.2.5 STATHMOKINETIC STUDY

Five subjects took part in this study, having given their consent after being fully informed of the procedures involved. The cell birth rate (k_B) was measured by two stathmokinetic techniques, cells being arrested by rapid intravenous infusion of vincristine sulphate in a dosage of 0.045 mg per kg body weight.

In the first procedure a peroral biopsy of proximal small-intestinal mucosa was taken from the region of the duodeno-jejunal junction at 1330 h on day 1, using a modified Crosby capsule. On day 2 the capsule was passed to the same position under radiological monitoring and a further biopsy obtained at the

same time as on the previous day, 2.5 h after the vincristine infusion. The birth rate for the crypt as a whole was estimated from the equation

$$k_B = \frac{I_m \text{ (day 2)} - I_m \text{ (day 1)}}{2.5}$$

and the crypt cell production rate (CCPR) by summing the birth rates at each cell position and multiplying by the column count.

In the second procedure the capsule of a Quinton multiple biopsy machine was passed to the same region as before; a biopsy was obtained, and after infusion of vincristine further biopsies were obtained at 15 minute intervals for about 2 h. The birth rate was estimated from the slope of a straight line fitted to the mitotic accumulation data, and the CCPR by considering once again the cumulative birth rates.

The handling, fixation, processing, and sectioning of the specimens was as in Section 6.3.2.1, and the method of counting as in Section 6.3.2.4, but with at least 3000 crypt-cell nuclei being counted for each biopsy specimen.

Three patients underwent the two-biopsy procedure:

AA, a male aged 61, suffered from rosacea and varicose ulcers of the legs. Both of his biopsy specimens were stereomicroscopically and histologically normal.

BB, a male aged 25, had a clinical diagnosis of DH but showed no clinical or biochemical evidence of malabsorption. Both of his biopsy specimens showed a completely convoluted mucosal surface on stereomicroscopy, while histological examination showed group-3-type changes.

CC, a male aged 37, had suffered from DH for over 10 years and had recently developed a megaloblastic anaemia due to folic acid deficiency; his skin condition was controlled by Dapsone and he was taking a normal diet. His mucosal biopsy specimens showed a flat avillous surface with a cobblestone appearance, and the histological changes were typical of untreated coeliac disease.

A further two patients were studied by the multiple biopsy procedure:

DD, a male aged 61, was being investigated to ascertain the cause of his chronic diarrhoea. Eleven biopsies, obtained over a period of 150 minutes, were considered to be morphologically normal.

EE, a female aged 55, had been diagnosed clinically as suffering from coeliac disease. Nine biopsies, obtained over 105 minutes, showed typical stereomicroscopic and histological appearances of untreated coeliac disease.

6.3.3 RESULTS

6.3.3.1 MORPHOMETRIC VARIABLES

The mean values for crypt length and column count in the 8 groups of patients studied are shown in Table 6.3.2 along with estimates of the total crypt-cell

population obtained from the product of these two measurements. The most marked contrast in the values both for crypt length and column count is between the controls and the groups with flat mucosae; the crypt lengths in all 3 such groups are more than double control values and are very similar to one another. Crypts over 100 cells long were seen in all coeliac-type groups. The column count in the adult coeliac disease group, however, is greater than in the two similar groups. The estimates of total crypt cell population show a threefold increase or more compared with the controls.

All three groups with convoluted mucosae have increased mean crypt length; this is present even in group 1, but is most marked in group 3 as would be anticipated from the definition of the groups (Table 6.3.1). In group-3-convoluted the column count is also noticeably increased, leading to a doubling of total crypt cell population compared with controls.

Mitotic index distributions are shown in Figure 6.3.4; positions of the HMP are marked. From these have been calculated the total cells below and above the HMP; we shall call these the proliferation and maturation zones although we cannot be sure that all the cells in the proliferation zone are in fact in cycle. Their populations are given in Table 6.3.2. The proliferation zone increases in

Table 6.3.2 Morphometric data for crypts in different clinico-morphological groups. All figures are cells

Patient group	Crypt length	Column count	Total cells	Proliferation zone	Maturation zone
Adult controls	32	25	800	550	250
Childhood controls	34	25	850	600	250
Group 1 convoluted	37	26	950	750	200
Group 2 convoluted	39	27	1050	750	300
Group 3 convoluted	49	31	1500	1150	350
Adult coeliac disease	74	41	3050	1700	1350
Childhood coeliac disease	75	35	2650	1550	1100
Dermatitis herpetiformis	76	36	2700	1650	1050

size in absolute terms in the convoluted groups, and also appears to be slightly greater as a proportion of the total population. In the groups with flat mucosae it increases even more in terms of cell numbers, but it is the maturation zone which shows the greater increase, having some five times more cells than in controls.

The I_m data also enable the CCPRs to be estimated if certain assumptions are made. Table 6.3.3 shows the mean numbers of mitoses observed per crypt column; if the duration of mitosis is 1 h then these are also the numbers of cells produced by a column every hour, and multiplication by the column counts give the CCPRs. However, since mitoses migrate towards the lumen of the crypt, they are seen preferentially in longitudinal sections (702), and so we have

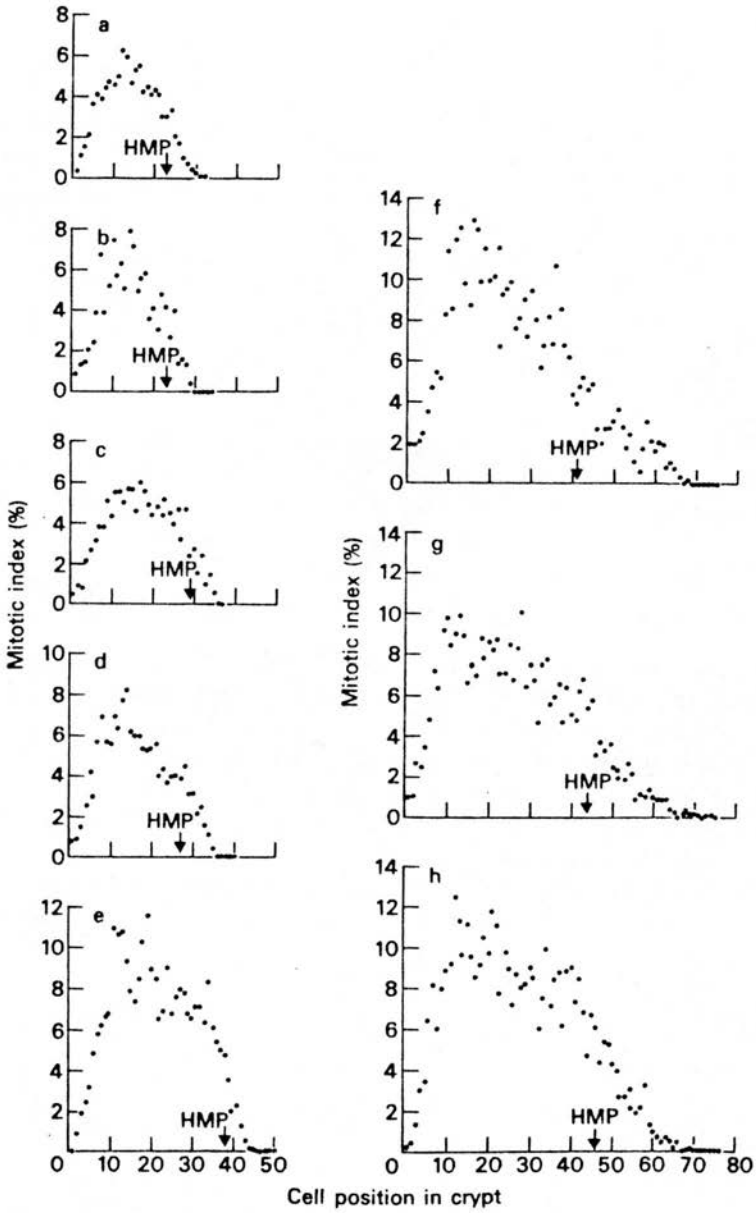

Figure 6.3.4 Mitotic index distribution in (a) adult controls (b) childhood controls (c) group 1 convoluted (d) group-2-convoluted (e) group-3-convoluted (f) adult coeliac disease (g) childhood coeliac disease and (h) dermatitis herpetiformis. Half-maximum positions are shown

Table 6.3.3 Observed number of mitoses per crypt column and estimated crypt cell production rate in different clinico-morphological groups

Patient group	Mitoses per column	CCPR (cells/crypt/h)
Adult controls	1.0	17
Childhood controls	1.2	21
Group 1 convoluted	1.3	23
Group 2 convoluted	1.5	28
Group 3 convoluted	2.7	59
Adult coeliac disease	3.9	110
Childhood coeliac disease	3.4	84
Dermatitis herpetiformis	4.1	105

multiplied the estimates of CCPR by 0.7 before entering them in Table 6.3.3. This value was obtained by examining crypt cross-sections in the samples from patients taking part in the stathmokinetic investigations; the effect of inaccuracy here or in the duration of mitosis can readily be seen.

6.3.3.2 STATHMOKINETIC STUDIES

The mitotic indices, corrected by multiplying by 0.7 as in Section 6.3.3.1, for the 3 patients subjected to the two biopsy procedure, are shown in Table 6.3.4 along with the cell birth rates and CCPRs derived from them.

Figure 6.3.5 illustrates the results of the multiple biopsy stathmokinetic technique on patients DD and EE, and Table 6.3.5 shows the birth rates calculated and the estimates of CCPR obtained from population sizes given in Table 6.3.2 for the appropriate groups.

The estimates of CCPR in all 5 stathmokinetic investigations show the same pattern between types of mucosa as do the estimates in Table 6.3.3 although

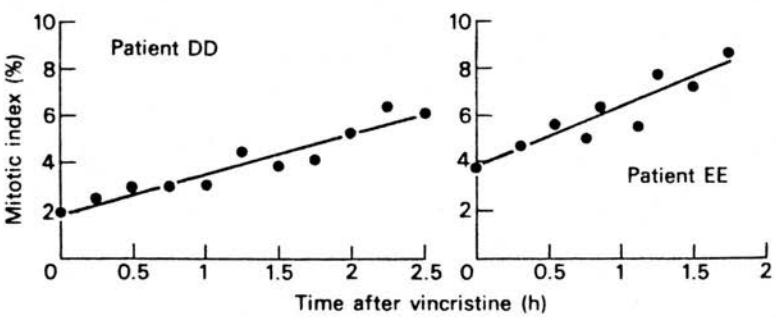

Figure 6.3.5 Stathmokinetic data from patients DD and EE

the stathmokinetic results are all slightly lower than would be expected from this table. A variety of reasons could account for this: failure of vincristine to arrest all cells in metaphase, degeneration of arrested metaphases, normal variability within or between individuals may all contribute to low values from stathmokinetic experiments. It is likely, however, that the values found by simple counting of mitoses are too high because the mitotic duration has been underestimated.

Table 6.3.4 Crypt size, mitotic index and cell production rate in 3 patients by a two-biopsy stathmokinetic technique

Patient	Patient group	Crypt cell population	Corrected I_m(%) Day 1	Day 2	k_B (cells/1000 cells/h)	CCPR (cells/crypt/h)
AA	Adult control	700	1.7	5.4	15	11
BB	Group 3 convoluted	1450	3.2	9.6	26	38
CC	Dermatitis herpetiformis	2050	3.6	10.4	27	55

Table 6.3.5 Birth rate in two patients by a multiple biopsy stathmokinetic technique. Crypt cell production rate is estimated from cell populations in Table 6.3.2

Patient	Patient group	k_B (cells/1000 cells/h)	CCPR (cells/crypt/h)
DD	Adult control	17	14
EE	Adult coeliac disease	25	76

6.3.4 DISCUSSION

6.3.4.1 MORPHOMETRIC PARAMETERS OF NORMAL VERSUS COELIAC CRYPTS

Simple inspection of well orientated histological sections suffices to show that coeliac crypts are longer than normal crypts, but it is less apparent that they are also increased in girth. If we compare the arithmetic means of the measurements for the two control groups and for the three coeliac groups respectively then we find that the mean crypt length is increased by a factor of 2.3 from 33 cells in the controls to 75 cells in the coeliacs. Similarly the mean column count is increased by a factor of 1.5 from 25 cells to 37 cells. The product of these two parameters gives a reliable estimate of the total crypt cell population (801) (Table 6.3.2), and if the mean values are compared we see that the total cell population of coeliac crypts is 3.4 times greater than in control crypts. The proliferation zone and the maturation zone both show an absolute increase in cell numbers, but the increase in size of the maturation zone is disproportionately greater than the increase in the size of the whole crypt.

6.3.4.2 PROLIFERATIVE PARAMETERS OF NORMAL VERSUS COELIAC CRYPTS

Despite the total atrophy and disappearance of villi from the proximal small-intestinal mucosa in untreated coeliac disease it is quite inappropriate to refer to the general state of the mucosa as one of atrophy; indeed the crypts are both hyperplastic and hyperproliferative. The assumption that this state constitutes a response to an abnormally high rate of cell loss, from the surface of the mucosa into the lumen of the bowel, was confirmed and quantified by Creamer and his colleagues (133, 526). They assessed the rate of cell loss by measuring the DNA content of the washings from perfused segments of small bowel and concluded that patients with untreated coeliac disease showed a sixfold increase over the normal rate of cell loss. Ordinarily the flat coeliac mucosa must achieve a new state of equilibrium to compensate for this change by a corresponding increase in the rate of cell production from the crypts. From the data in Table 6.3.3 it is evident that the CCPR from coeliac crypts is 5.2 times greater than from normal crypts. If the comparison is restricted to adult controls and adult coeliacs the increase in CCPR is 6.5 times, an estimate which matches closely the estimated increase in the rate of cell loss. These comparisons are valid only if we assume that there is no significant increase in the number of crypts per unit length of small bowel in coeliacs as compared with normals.

The increases in CCPR can be partly accounted for by the near trebling of cell number in the proliferation zone of the crypt, and for the rest by an approximately 50 percent reduction in cell cycle time, always assuming that t_m remains virtually constant. The results from the stathmokinetic studies are similar though lower (Tables 6.3.4 and 6.3.5) and possible explanations for this slight discrepancy have already been mentioned.

6.3.4.3 SIGNIFICANCE OF A CONVOLUTED MUCOSA

The duodeno-jejunal mucosa can assume a flat avillous appearance, with crypts opening directly or through shared vestibular chambers on to the surfaces of a mosaic of low plateaux, in conditions other than coeliac disease (358). Nevertheless, these changes are so characteristic as to be virtually diagnostic of untreated coeliac disease in appropriate clinical circumstances. On the other hand a convoluted appearance is often of unknown significance as an isolated finding even when all other aspects of the case have been taken into account (768, 817). In the context of coeliac disease a convoluted appearance represents an intermediate stage in the recovery or in the development of a flat mucosa. The distal limit of a flat coeliac mucosa may be separated from unaltered villous mucosa by an intermediate convoluted zone. Islands of convoluted mucosa may be found in an otherwise flat coeliac mucosa by multiple sampling. Convolutions may be a feature of various other disorders and the prevalence in apparently healthy subjects differs between population groups (33, 452, 817).

Our studies of the morphometric and proliferative parameters show that group-3-convoluted mucosae are in a hyperproliferative state intermediate between normal and flat mucosae; groups 1 and 2, on the other hand, differ little from normal controls. Our data are consistent with the hypothesis that group-3-convoluted mucosae represent a morphological adaptation to a moderately reduced population of villous enterocytes consequent upon increased cell loss and balanced by an appropriate increase in cell production. If the rate of cell loss increases further, the preservation of surface integrity entails loss of convolutions (so-called 'total-villous atrophy') and further stepping up of cell production perhaps to near maximum rates.

6.3.4.4 MECHANISMS FOR INCREASING CCPR IN RESPONSE TO INCREASED RATE OF LOSS OF MATURE ENTEROCYTES

In the light of present knowledge we can reasonably discard certain suggestions some of which are now of little more than historical interest. These include *mitotic arrest* as a cause of the increased mitotic index in coeliac crypts (598); though experimental models based on this concept show villous atrophy, there is also crypt atrophy (108). The concept of *enteroblastic hypoplasia* (131) is not in keeping with the usual finding of an increased mitotic index in flat coeliac mucosae; the patient shown by tritiated-thymidine (^{3}HTdR) studies to have a reduced output of cells from the crypts must be regarded as atypical (131). *Maturation arrest or delay* (131, 132) could in theory account for a reduction in CCPR even in the face of an increase in the rate of cell division within the proliferation zone. This would require loss or death of cells in the maturation zone and for this there is no morphological evidence (601). Furthermore, studies of coeliac mucosae maintained in organ culture have convincingly shown an increased labelling index when ^{3}HTdR was added to the culture medium, and an increase in the rate of migration from the crypts (724). In terms of Booth's analogy with the haemopoietic system (52, 53) our studies support the concept of *enteroblastic hyperplasia* comparable to erythroblastic hyperplasia in haemolytic anaemia. This reaction in the small-intestinal mucosa is mediated firstly by an increase in the size of the crypt with an absolute increase in the population of proliferating cells, and secondly by a reduction in T_c to about half its normal value. Whether these compensatory changes occur simultaneously or sequentially, and if the latter in what order, remains to be elucidated.

ACKNOWLEDGEMENT

The work reported in this paper was supported by a grant from the North of England Council of the Cancer Research Campaign. We are grateful to clinical colleagues, in particular to Dr. Janet Marks, for their collaboration.

David Appleton

Adaptive Cell-proliferative Changes in the Small-intestinal Mucosa in Coeliac Disease

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Cell proliferation in the small-intestinal crypts of rodents has been intensively investigated and lends itself to the deployment of techniques which are inapplicable in man. In particular there are ethical and economic objections to methods involving the use of tritiated thymidine in vivo for specific labelling of DNA, while the validity of in vitro studies using organ culture is uncertain. It is possible, nevertheless to construct a profile of the size and cytokinetic status of the mucosal crypts by analysis of serial sections prepared from well orientated routine diagnostic biopsy specimens. Such studies can provide a measure of mean total crypt-cell population, and by studying the distribution of mitoses in the crypts relative measurements can be obtained of proliferation and maturation compartment sizes, and of crypt cell production rate (CCPR). These parameters have been compared in 62 patients with 'flat' avillous coeliac mucosae and in 85 patients with normal villous mucosae. A heterogeneous group of 47 patients with lesser degrees of abnormality (convoluted mucosae) were similarly studied. In addition, estimates of cell cycle times were obtained in a small group of patients with normal, convoluted and 'flat' mucosae by taking biopsies before and after the administration of the metaphase-arresting agent vincristine. 'Flat' coeliac mucosae show a threefold increase in the size of the proliferation compartment compared with normal and the cell cycle time is approximately halved leading to a net sixfold increase in CCPR. This is the basis of the change in mucosal morphology and presumably represents a compensatory reaction to the gluten-induced increase in loss of enterocytes from the mucosal surface. Convoluted mucosae occupy an intermediate position in terms of the parameters studied and should be regarded as stages in a continuum of adaptive change.

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INTRODUCTION

Coeliac disease was first recognised as a clinical entity defined in terms of its symptoms and signs, with emphasis on evidence of malabsorption and in particular of steatorrhoea. In common with many other syndromes its characterisation as a specific nosological entity could be achieved only when a specific aetiological agent was identified (19). It is now well established that the condition of overt coeliac disease occurs in susceptible individuals whose diet includes products prepared from the grain of wheat, rye, barley and oats. The starch fraction of the flour from these grains is innocuous, but the heterogeneous alcohol-soluble component (gliadin) of the protein fraction (gluten) was soon shown to be capable of inducing the condition (20) and this harmful property was still present in a water-soluble peptide fraction produced by peptic-tryptic digestion of wheat gliadin (26). However, it has not yet proved possible to identify the responsible factor precisely, at molecular level, despite intensive investigation. The first reliable demonstration of the morphological changes in the small-intestinal mucosa in untreated coeliac disease was based on the examination of specimens obtained fortuitously at laparotomy (39). Subsequently the development of peroral suction-biopsy techniques (18, 25, 44, 50) allowed the characteristic histopathological features to be fully documented (1, 21, 47, 60) together with the sequence of changes by which

normal morphology is restored during successful treatment, and the mode of reversion when treatment is discontinued (49). Additional important information has also been derived from the application of techniques such as stereomicroscopic examination with the dissecting microscope (27, 45) and the more sophisticated scanning electron microscope (3, 35, 36). The nature of the cellular and subcellular changes affecting the enterocytes, i.e. the monolayer of closely packed columnar epithelial cells which normally cover the villi, and whose residual population of shortened, pseudostratified cells cover the surface of the avillous coeliac mucosa, have been intensively studied by enzyme histochemistry (43, 54), by transmission electron microscopy (48, 51) and by analytical subcellular fractionation techniques (41).

The first hypothesis propounded to explain the pathogenesis of coeliac disease in terms of gluten sensitivity stipulated the constitutional absence of some specific enzyme, possibly a peptidase, normally present within the surface epithelium. Short-term organ-culture studies appeared to indicate that gluten or gliadin digests had no direct deleterious effect on treated coeliac mucosa in vitro (22, 29). Recently, however, it has been reported that gluten-sensitivity can be demonstrated during organ culture not only in untreated, but also in treated coeliac mucosa by actual quantitative assessment of changes in enterocyte height (28). Peters, Jones & Wells (41) found a persistent defect in brush-border enzyme activities in morphologically normal coeliac mucosa after prolonged gluten withdrawal; this defective activity was particularly marked for beta-glucosidase. The concept of a primary enzyme deficiency cannot yet be regarded as entirely abandoned.

The weight of current opinion tends to favour the major alternative explanation which postulates that the harmful effects of gluten are mediated by immunological mechanisms (38). Of course these two hypotheses are not necessarily mutually exclusive, and if there is an inherited abnormality in the coeliac enterocyte it may be concerned with surface receptors (38) rather than enzymes. Although the role of these mechanisms is not fully understood, and it is probable that local humoral activity is not central to the pathogenesis (37) there are indications that local cell-mediated immune reactions to gluten may be involved (8, 23, 24, 32). Marsh's report (37) that a high mitotic index in the population of intraepithelial lymphocytes in untreated coeliac disease is specifically indicative of a state of gluten-sensitive enteropathy is of great theoretical interest and is likely to be of practical importance.

The difficulties in achieving an acceptable definition of coeliac disease have been pithily expressed by Creamer (16), but we suggest for most practical purposes that it be regarded as a malabsorptive condition resulting from a probably permanent state of intolerance to dietary gluten associated with a flat avillous duodenojejunal mucosa on stereomicroscopy, usually with a mosaic pattern, and with characteristic histological changes, in particular, total villous atrophy, crypt hyperplasia and a marked infiltration of the lamina propria by plasma cells and macrophages. Strict adherence to a gluten-free diet is followed in some 85% of cases by clinical and morphological improvement, but there is a liability to relapse following reintroduction of gluten. If the finding of mitoses amongst intraepithelial lymphocytes is as diagnostically discriminatory as Marsh (37) asserts, this will help to overcome the difficulty presented by the occasional finding of a 'flat' pseudocoeliac mucosa in a variety of unrelated conditions; it will also obviate the need for second and third biopsies to check response to treatment and reversion of the improvement on gluten challenge.

It is the purpose of this paper to present an integrated account of the proliferative status of the crypt-cell population in the 'flat' avillous small-intestinal mucosa of untreated coeliac disease and to attempt to correlate this with the altered morphology of the crypts and villi. A comparison will be drawn with the findings in a series of morphologically normal mucosae and it will also be established that mucosae with intermediate morphological abnormalities probably represent stages in a continuum of increasingly pronounced cytokinetic adjustments. This present account incorporates results published elsewhere (58, 60, 61, 64, 65, 66, 67, 68). Despite the

necessary limitations related to ethical and technical considerations we believed that it was necessary to carry out a direct study of human material; this has been conducted mainly by analysis of routine diagnostic biopsies with the exception of a few instances in which a stathmokinetic technique was applied in a small group of fully informed volunteers.

MATERIALS AND METHODS

Patients

In most instances a single biopsy sample of proximal small-intestinal mucosa was obtained by the peroral route, primarily for diagnostic purposes, usually to confirm or to exclude a provisional diagnosis of coeliac disease. Other mucosal biopsies were obtained from patients, with psoriasis (52) or other dermatoses, in whom the presence of dermatogenic enteropathy was being investigated (33). During the course of such studies the association between dermatitis herpetiformis (DH) and coeliac disease was discovered (34, 53, 59); in this association the coeliac condition is usually clinically latent.

A total of 62 patients had a 'flat' coeliac-type mucosa; this group comprised 13 adults and 29 children with untreated coeliac disease and 20 adults with dermatitis herpetiformis. Morphologically normal biopsy specimens were obtained from 85 patients (75 adults and 10 children) and these mucosal samples constituted our control series. A further series of 47 biopsy specimens, designated 'convoluted' mucosae for reference purposes, showed an intermediate range of abnormalities (Table I). The patients from whom these mucosal samples were obtained constituted a heterogeneous group in term of clinical diagnosis, but it is noteworthy that 15 had DH and 2 were first degree relatives of DH patients. Six were coeliac patients in various stages of remission or relapse, and 4 were suffering from giardiasis.

Methods

Peroral biopsy specimens were taken under radiological control from the vicinity of the duodeno-jejunal junction using a modified Crosby suction-biopsy capsule. The capsule was immediately withdrawn and the specimen retrieved without delay; it was then orientated under a dissecting microscope and gently spread, mucosal surface upwards, on to a thin piece of glass. Primary fixation was 10% neutral buffered formol saline and detailed stereomicroscopic examination was postponed for at least 24 hours, when obscuring mucus and debris could be removed by gentle brushing without risk of damage. In order to improve the quality of fixation and to give better nuclear staining, the specimen

Table I. Synopsis of changes which characterise convoluted mucosae assigned to each of three arbitrarily defined groups.

Group	Stereomicroscopy	Histology
1	Not more than a few convolutions, distributed amongst leaves and ridges, and occupying less than 10% of mucosal surface	No notable abnormality
2	From 10 to 70% of mucosal surface occupied by convolutions	Some shortening of villi evident on inspection; little or no apparent lengthening of crypts; surface epithelium normal; density of inflammatory cell infiltration normal or just perceptibly increased
3	Mucosal surface completely or predominantly (over 70%) convoluted	Short stubby villi; crypts obviously lengthened; surface epithelium abnormal; obvious increase in density of inflammatory cell infiltration

was post-fixed in a 5% solution of mercuric chloride in formol saline; it was then processed for embedding in vertical orientation in paraffin wax. Approximately 200 serial sections, cut at a thickness of 3 μm and stained by Harris's haematoxylin, were used for the purpose of crypt analysis (see below). For immediate diagnostic assessment several sections were stained by haematoxylin and eosin and by the periodic-acid Schiff method after amylase digestion; 'flat' coeliac mucosae were also routinely stained by the haematoxylin-van Gieson and by the picro-Mallory methods to detect collagenisation of the lamina propria.

Morphological classification

The biopsy specimens were assigned to one of three main categories on the basis of their morphological appearances as seen by stereomicroscopic examination under the dissecting microscope and by conventional light microscopy of histological sections. Although the use of stereomicroscopy has been decried (31, 40, 46) it remains a valuable adjunct to histological examination and should not be neglected.

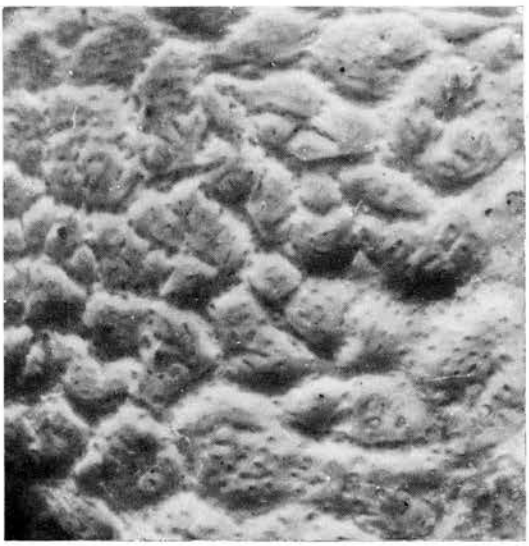

Fig. 1. A peroral biopsy of proximal small-intestinal mucosa from a patient with untreated coeliac disease. The 'flat' avillous mucosal surface shows a characteristic mosaic pattern. Some of the hyperplastic crypts open directly on to the surface, but it is more usual for several crypts to open into short communal vestibules whose orifices are dispersed over the surface. (× 12) (From Watson et al., 58).

Fig. 2. This shows the characteristic histological findings in an untreated coeliac mucosa. There is total villous atrophy whereas the crypts are hyperplastic; two crypts are seen opening into a shared vestibule; dividing crypt cells are plentiful and occur to a high level in the crypts, the lamina propria shows a heavy infiltration of chronic inflammatory cells; there are numerous lymphocytes in the surface epithelium which also shows nuclear pseudostratification. (Haematoxylin and eosin × 150) (From Watson et al., 58).

'Flat', avillous, coeliac-type mucosae. A characteristic stereomicroscopic appearance is seen (Fig. 1) of which the most significant feature is the complete absence of any projecting structures which could conceivably merit the term villi. The adjective 'flat' is somewhat inappropriate, but is in general use and its connotation is unambiguous. The corresponding histological features are equally characteristic (Fig. 2).

Normal mucosae. These specimens showed a normal villous appearance on stereomicroscopy and conformed to the conventional appearance of normality on histological examination. In adults especially it is relatively unusual to find mucosa from the region sampled showing exclusively a population of tall finger-shaped villi; narrow or broad leaf-shaped forms were accepted as normal, and the presence of curved or angular

leaves, of joined leaves, of straight or sinuous ridges amonst the more regular leafy villi did not exclude specimens from this category. But specimens showing convolutions were invariably excluded.

Convoluted mucosae. Specimens in this category are most easily and reliably identified when the various forms of villous structure described above have been wholly replaced by a complex of intertwining flexuous ridged formations (Fig. 3) reminiscent of the cerebral convolutions; the height of the convolutions often appears comparable to that of normal villi. For our purposes we have included all mucosae with convolutions in this category irrespective of the prevalence of these forms and it is clear, therefore, that this is a heterogeneous group. It is heterogeneous also in respect of the histological appearances which range from normality through varying degrees of villous atrophy, crypt hyperplasia, with inflammatory and other changes eventually approaching in severity those of untreated coeliac disease. Indeed, markedly atrophic forms of convolutions are occasionally seen which are more appropriately regarded as variants of 'flat' mucosae. Consequently we have divided the convoluted mucosae into three subgroups on the basis of increasing severity of morphological abnormality according to arbitrary criteria (Table I). In those instances where there was disparity between the extent of the convoluted state and the degree of accompanying histological abnormality, the specimen was assigned to its subgroup on the basis of the more marked alteration.

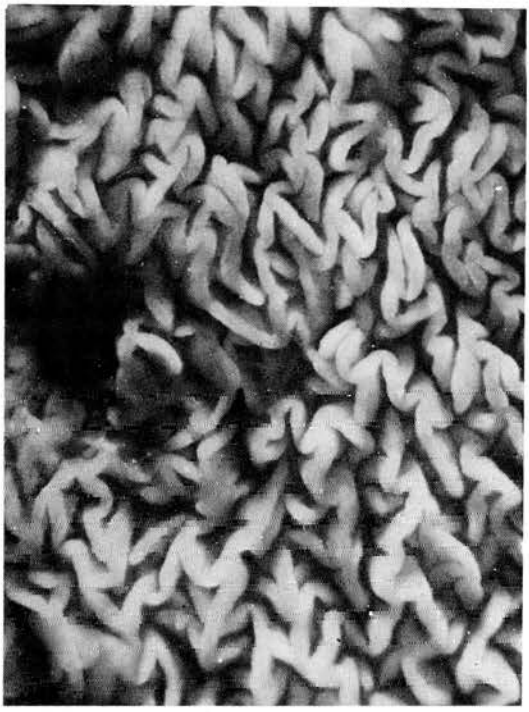

Fig. 3. A peroral biopsy of proximal small-intestinal mucosa as viewed through one eyepiece of a dissecting microscope; after fixation the epithelium is no longer transparent. Villi have been replaced by so-called convolutions and this specimen would be assigned to group 3. (× 12) (From Watson, Wright & Appleton, 58).

Methods of analysis

In all of the morphological categories the aim was to analyse thirty separate crypts, each of which had been sectioned in its longitudinal axis so as to include the base, middle and mouth in the plane of the histological section. Crypt length was measured in terms of cell units by enumerating the 'left-hand' column of cells from the bottom to the mouth of the crypt as seen in section. In villous mucosae the mouth corresponds approximately to the plane of the crypt villous junction, whereas in avillous coeliac mucosae the mouth is represented by the apparent junction of the crypt with the surface epithelium (Fig. 2). Within the crypt column the positions of all cells with mitotic nuclei were recorded and, by pooling the data for all crypts examined in any group of mucosae, the distribution of the mitotic index (I_m) values at each cell position, in a crypt with a length equal to the mean for the appropriate group, was determined (9, 63).

A useful description of the extent of cell proliferation up the length of the crypt is the so-called cut-off position (10, 11, 55) at which cells in effect receive a signal to complete the present cycle, but not to enter a further cycle. We have described elsewhere (2) how this can be found from the results of stathmokinetic experiments provided an estimate of the cell cycle time is available. As this requires determination of the frequency of labelled mitoses (FLM) following injections of tritiated thymidine we have been

unable to ascertain the cut-off position in our human material. Instead, we have derived from the mitotic index distribution curves, estimates of the cell position at which I_m falls to 50% of its maximum value (13); we term this the half-maximum position (HMP). In the hypothetical situation in which all cells below this position were proliferating with a cell cycle time of equal duration then the cut-off position would be half this distance from the bottom of the crypt.

Stathmokinetic study

Five patients participated in this study having given their informed consent after receiving a full explanation of the procedure involved. The project was also approved by the Ethical Committee of the Newcastle University Hospital Group. Two procedures were used to permit measurement of the crypt-cell birth rate (k_B) after inducing stathmokinesis by rapid intravenous infusion of vincristine sulphate in a dosage of 0.045 mg per kg body weight.

Three patients underwent a two-biopsy procedure. On day 1 a peroral biopsy of proximal small-intestinal mucosa was taken from the region of the duodeno-jejunal junction at 13:30h, using a modified Crosby capsule whose position was monitored radiologically. On day 2 a second biopsy was obtained from the same position at the same time of day, exactly 2.5h after the vincristine infusion. The birth rate for the whole crypt was estimated from the equation

$$k_B = \frac{I_m\,(day\ 2) - I_m\,(day\ 1)}{2.5}$$

and a value for the crypt-cell production rate (CCPR) was obtained from the sum of the birth rates at each cell position in a single crypt column multiplied by the total number of columns in the crypt i.e. the column count.

Patient AA was a man aged 61 with a history of rosacea and varicose ulcers of the legs. Both biopsy specimens showed finger-shaped and narrow leaf-shaped villi and appeared histologically normal.

Patient BB was a man aged 25 suffering from dermatitis herpetiformis; he presented no clinical or biochemical evidence of malabsorption. Both biopsy specimens showed a completely convoluted surface associated with histological changes of group 3 severity.

Patient CC was a man aged 37 with a history of DH for ten or more years, who had recently developed a megaloblastic anaemia due to folic acid deficiency: his skin condition was controlled by Dapsone and he was taking a normal diet. His biopsy specimens both showed a 'flat' avillous appearance with histological changes indistinguishable from those of untreated coeliac disease.

Another two patients were studied by a multiple biopsy procedure using a Quinton multiple biopsy machine. The biopsy capsule was passed to the region of the duodeno-jejunal junction as before and a sample of mucosa retrieved; after intravenous infusion of vincristine further specimens of mucosa were retrieved at approximately 15 minute intervals over a period of about 2 h in one instance and 2.5 h in the other. The cell birth rate for the whole crypt was estimated from the slope of a straight line fitted to the mitotic accumulation data; as before the CCPR was obtained from consideration of the cumulative birth rates at each cell position.

Patient DD was a man aged 61 who was being investigated to ascertain the cause of his chronic diarrhoea. Eleven biopsies, obtained over a period of 150 minutes, were considered to be morphologically normal.

Patient EE was a woman aged 55 in whom a provisional clinical diagnosis of coeliac disease had been made. Nine biopsies, obtained over 105 minutes, showed a 'flat' avillous appearance and characteristic histological changes of untreated coeliac disease.

The handling, fixation, processing and sectioning of all these specimens was exactly as described above for single biopsies; crypt analysis was also essentially similar but even more meticulous in that at least 3000 crypt-cell nuclei were counted for each biopsy specimen.

RESULTS

Morphometric variables

The mean values for crypt length and column count in the eight groups of patients studied are

Table II. Cell population data for crypts in different clinical/morphological groups. ACD = adult coeliac disease; CCD = childhood coeliac disease; DH = dermatitis herpetiformis. All figures are for cells

Mucosa	Patients	Crypt length	Column count	Total cells	Proliferation zone	Maturation zone
Normal	Adults (75)	32	25	800	550	250
	Children (10)	34	25	850	600	250
Convoluted:						
group 1 (15)		37	26	950	750	200
group 2 (18)		39	27	1050	750	300
group 3 (14)		49	31	1550	1150	350
'Flat' avillous	ACD (13)	74	41	3050	1700	1350
	CCD (29)	75	35	2650	1550	1100
	DH (20)	76	36	2700	1650	1050

shown in Table II together with estimates of the total crypt-cell population obtained from the product of these two measurements. The most marked contrast in the values both for crypt length and column count is between the normal controls and the groups with flat mucosae; all of the latter have crypt length values which are more than double those of the controls, and all include crypts more than 100 cells long. Column counts are greatly increased in the coeliac-type groups, but especially in adult coeliacs. The estimates of total crypt-cell population show a threefold or greater increase compared with controls.

All three groups with convoluted mucosae have an increased mean crypt length, though the increase is greatest in group 3 as might be anticipated from the definition of these groups (Table I). In group 3 the column count is also clearly increased and consequently the total crypt-cell population is double that of the controls.

The location of the half maximum position (HMP) can be identified from the mitotic index distributions (Fig. 4) and we refer to the total population of cells below this position as occupying the 'proliferation zone' although it is possible that some of the cells in this zone are not in cycle. The zone above the HMP is termed the 'maturation zone'. The sizes of these populations in the different groups are shown in Table II. In the convoluted mucosae the proliferation zone increases in size in absolute terms and also appears to be slightly increased as a proportion of the whole population. The flat mucosae show an even greater increase in the proliferation zone

Table III. Observed number of mitoses per crypt column and estimated crypt cell production rate (CCPR) in the different clinical/morphological groups. Abbreviations as in Table II

Mucosa	Patients	Mitoses per column	CCPR (cells/crypt/h)
Normal	Adults (75)	1.0	17
	Children (10)	1.2	21
Convoluted:			
group 1 (15)		1.3	23
group 2 (18)		1.5	28
group 3 (14)		2.7	59
'Flat' avillous	ACD (13)	3.9	110
	CCD (29)	3.4	84
	DH (20)	4.1	105

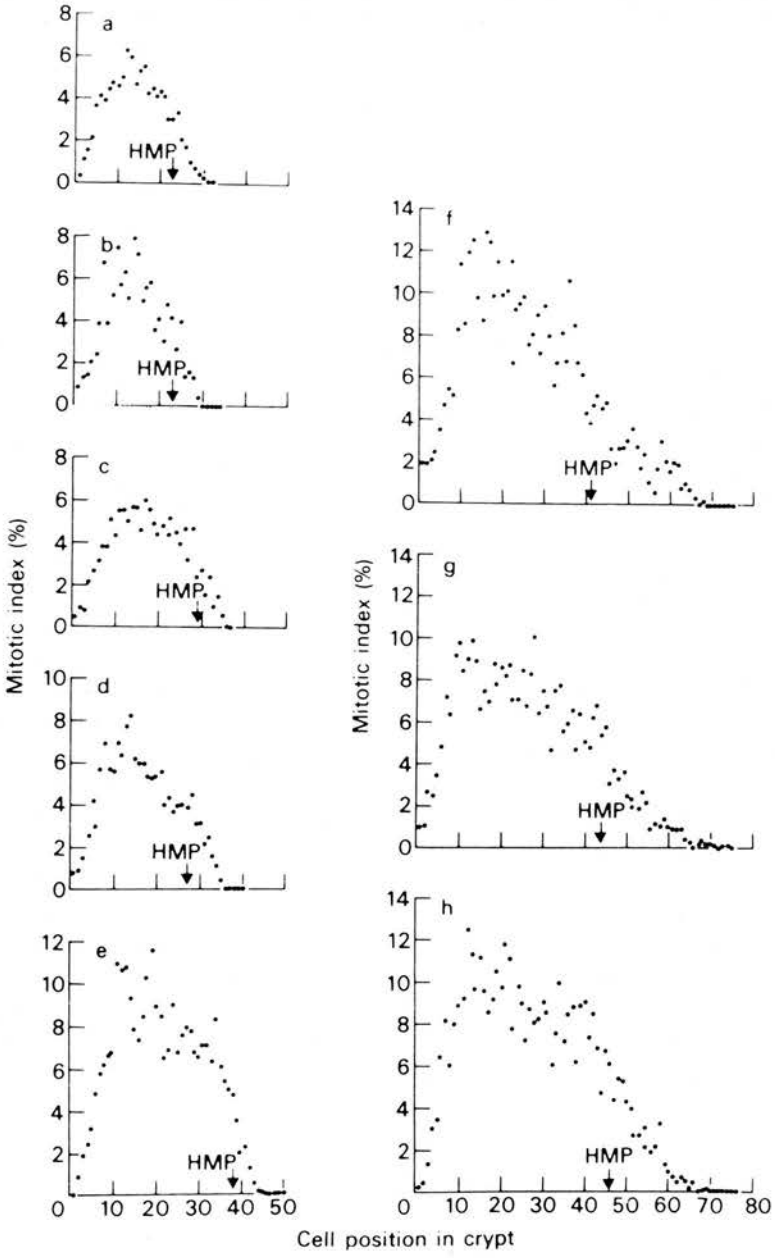

Fig. 4. Mitotic index distributions in (a) adult controls, (b) childhood controls (c) group-1 convoluted, (d) group-2 convoluted, (e) group-3 convoluted, (f) adult coeliac disease, (g) childhood coeliac disease and (h) dermatitis herpetiformis. Mitotic indices (%) are indicated on the vertical axes and cell position in the crypt on the horizontal axes. Half-maximum positions (HMP) are shown. (From Watson et al., 58).

Table IV. Data relating to three patients studied by the two-biopsy stathmokinetic technique. The mitotic index has been corrected for the size of the proliferation zone; k_B = birth rate at top of crypt; CCPR = crypt cell production rate

Patient	Category	Crypt cell population	Corrected mitotic index (%) Day 1	Day 2	Birth rate (k_B) (cells/1000 cells/h)	CCPR (cells/crypt)h)
AA	Adult control	700	1.7	5.4	15	11
BB	Group 3 convoluted	1450	3.2	9.6	26	38
CC	Dermatitis herpetiformis	2050	3.6	10.4	27	55

in terms of cell numbers, but the size of the increase is exceeded in the maturation zone which has some five times more cells than in the controls.

Provided certain assumptions are made it is possible to estimate the crypt-cell production rates from the I_m data. Table III shows the mean numbers of mitoses observed per crypt column; if the duration of mitosis is taken as 1 h, then these are also the numbers of cells produced by a column every hour and multiplication by the column counts will give values for the CCPR. Because mitotic nuclei are found nearer the central axis of the crypt than intermitotic nuclei Tannock (56) pointed out that they tend to be over-represented in longitudianl sections and that this leads to an overestimate of the mitotic index. It is necessary to apply a correction factor which can be derived from the ratio of the mean radial displacement of mitotic nuclei from the central axis, to the corresponding mean distance for intermitotic nuclei. We derived a correction factor of 0.7, applicable both to normal and to abnormal mucosae, by appropriate measurements in histological cross-sections of the mucosal samples of patients taking part in the stathmokinetic studies. The estimates of CCPR entered in Table III have been reduced by this factor; it can readily be seen that any inaccuracies in the estimate of mitotic duration or failure to apply Tannock's correction factor will be reflected in a similar loss of accuracy in estimating CCPR.

Stathmokinetic observations

The mitotic indices, corrected by multiplying by 0.7 as explained above, for the patients subjected to the two-biopsy procedure are shown in Table IV along with the cell birth rates and the CCPRs derived from them.

The results of the multiple biopsy stathmokinetic technique on the two patients DD and EE are illustrated in Figure 5 and Table V shows the calculated birth rates together with estimates of CCPR obtained from the population sizes for the appropriate groups as given in Table II.

The estimates of CCPR in the five stathmokinetic investigations all show the same general pattern of variation between the different morphological types of mucosa as do the estimates

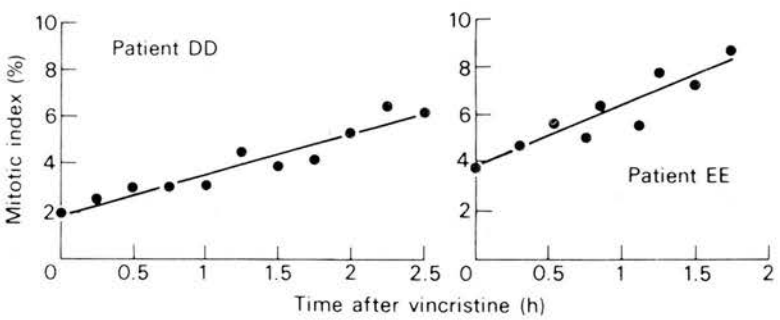

Fig. 5. Stathmokinetic data from patients DD and EE. (From Watson et al., 58).

Table V. Data relating to two patients studied by the multiple biopsy stathmokinetic technique. Crypt cell production rate is estimated from cell populations of corresponding categories in Table II

Patient	Category	Birth rate (cells/1000 cells/h)	CCPR (cells/crypt/h)
DD	Adult control	17	14
EE	Adult coeliac	25	76

in Table III although the stathmokinetic results are all slightly lower than would be expected. Amongst the reasons which might account for this are failure of vincristine to arrest all cells in metaphase, degeneration of arrested metaphases, and normal variability within or between individuals. On the other hand it is likely that the values found by simple enumeration of mitoses are too high because the mitotic duration has been underestimated.

DISCUSSION

Normal versus coeliac crypts

Morphometric parameters. Simple inspection of well orientated histological sections suffices to show that coeliac crypts are longer than normal crypts, but is less apparent that they are also increased in girth. If we compare the arithmetic means of the measurements for the two control groups and for the three coeliac groups respectively then we find that the mean crypt length is increased by a factor of 2.3 from 33 cells in the controls to 75 cells in the coeliacs. Similarly the mean column count is increased by a factor of 1.5 from 25 cells to 37 cells. The product of these two parameters gives a reliable estimate of the total crypt cell population (62) (Table II), and if the mean values are compared we see that the total cell population in coeliac crypts is 3.4 times greater than in control crypts. The proliferation zone and the maturation zone both show an absolute increase in cell numbers, but the increase in size of the maturation zone is disproportionately greater than the increase in size of the whole crypt.

Proliferative parameters. Despite the total atrophy and disappearance of villi from the proximal small-intestinal mucosa in untreated coeliac disease it is quite inappropriate to refer to the general state of the mucsoa as one of atrophy; indeed the crypts are both hyperplastic and hyperproliferative. The assumption that this state constitutes a response to an abnormally high rate of cell loss, from the surface of the mucosa into the lumen of the the bowel, was confirmed and quantified by Creamer and his colleagues (17, 42). They assessed the rate of cell loss by measuring the DNA content of the washings from perfused segments of small bowel and concluded that patients with untreated coeliac disease showed a six-fold increase over the normal rate of cell loss. Ordinarily the 'flat' coeliac mucosa must achieve a new state of equilibrium to compensate for this change by a corresponding increase in the rate of cell production from the crypts. From the data in Table III it is evident that the CCPR from coeliac crypts is 5.2 times greater than from normal crypts. If the comparison is restricted to adult controls and adult coeliacs the increase in CCPR is 6.5 times, an estimate which matches closely the estimated increase in the rate of cell loss. These comparisons are valid only if we assume that there is no significant increase in the number of crypts per unit length of small bowel in coeliacs as compared with normals.

The increase in CCPR can be partly accounted for by the near trebling of cell number in the proliferation zone of the crypt, and for the rest by an appproximately 50 per cent reduction in cell cycle time, always assuming that the duration of mitosis remains virtually constant. The results from the stathmokinetic studies are similar though lower (Tables IV and V) and possible explanations for this slight discrepancy have already been mentioned.

Significance of a convoluted mucosa

The duodeno-jejunal mucosa can assume a 'flat' avillous appearance, with crypts opening directly or through shared vestibular chambers on to the surfaces of a mosaic of low plateaux, in conditions other than coeliac disease (30). Nevertheless, these changes are so characteristic as to be virtually diagnostic of untreated coeliac disease in appropriate clinical circumstances. On the other hand a convoluted appearance is often of unknown significance as an isolated finding even when all other aspects of the case have been taken into account (60, 61, 64). In the context of coeliac disease a convoluted appearance represents an intermediate stage in the recovery or in the development of a 'flat' mucosa. The distal limit of a 'flat' coeliac mucosa may be separated from unaltered villous mucosa by an intermediate convoluted zone. Islands of convoluted mucosa may be found in an otherwise 'flat' coeliac mucosa by multiple sampling. Convolutions may be a feature of various other disorders and the prevalence in apparently healthy subjects differs between different population groups (4, 33, 64).

Our studies of the morphometric and proliferative parameters show that group-3 convoluted mucosae are in a hyperproliferative state intermediate between normal and 'flat' mucosae whereas groups 1 and 2 differ little from normal controls. Our data are consistent with the hypothesis that group-3 convoluted mucosae represent a morphological adaptation to a moderately reduced population of villous enterocytes consequent upon increased cell loss and balanced by an appropriate increase in cell production. If the rate of cell loss should increase further, then preservation of surface integrity would entail loss of convolutions leading to total villous atrophy and further stepping-up of cell production, perhaps to near maximum rates.

Nature of adaptive responses

In the light of present knowledge we can reasonably discard certain suggestions some of which are now of little more than historical interest. These include mitotic arrest as a cause of the increased mitotic index in coeliac crypts (45); though experimental models based on this concept show villous atrophy they differ from coeliac disease in that this change is accompanied by crypt atrophy (12). The concept of enteroblastic hypoplasia (15) is not in keeping with the characteristically increased mitotic index in flat coeliac mucosae; the patient shown by in vivo tritiated-thymidine studies to have a reduced output of cells from the crypt must be regarded as atypical (14). In theory, maturation arrest or delay might account for a reduction in CCPR, but this explanation can be accommodated only if we also postulate loss or death of cells in the maturation zone; there is no morphological evidence for this occurrence (48). Furthermore, studies of coeliac mucosae maintained in organ culture have convincingly shown an increased labelling index compared to normal when tritiated-thymidine was added to the culture medium, and an increase in the rate of migration from the crypts (57). In terms of an analogy with the haemopoeitic system (5, 6, 7) our studies support the concept of an enteroblastic hyperplasia in the coeliac mucosa comparable to the erythroblastic hyperplasia which occurs in haemolytic anaemia. In the small-intestinal mucosa this response is mediated by a threefold increase in the population of proliferating crypt cells, an increase which entails considerable enlargement of the length and girth of the crypts to accommodate these and the enlarged population of maturing cells. Also contributing to the roughly sixfold increase in the rate of output of cells from the crypts is a reduction in cell cycle time to about half its normal value. Whether these compensatory changes occur synchronously, or to some greater or lesser extent sequentially, and if the latter in what order of sequence, cannot be ascertained from our observations or from those of any currently available, acceptable techniques.

ACKNOWLEDGEMENTS

The work reported in this paper was supported by a grant from the North of England Council of the Cancer Research Campaign. Figs. 1 to 5 are reproduced in whole or in part from the publication cited as reference (58), and Tables I to V have been adapted from the same source, with

the permission of the publishers Pitman Medical. The authors are grateful to Miss E. Wark for secretarial assistance.

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J. Anat. (1982), **134**, 3, pp. 459–469
With 7 figures
Printed in Great Britain

Crypt regeneration in adult human colonic mucosa during prolonged organ culture

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(*Accepted* 28 *April* 1981)

INTRODUCTION

Samples of human colonic mucosa from normal individuals and from patients with various non-neoplastic diseases of the large bowel have been studied previously in short term organ culture, i.e. up to 48 hours, using histochemical, biochemical and cytokinetic techniques (Eastwood & Trier, 1973*a*, *b*; MacDermott, Donaldson & Trier, 1974; Trier, 1976; Neutra, Grand & Trier, 1977). Although neoplastic mucosa has been maintained *in vitro* for long periods (Roller, Owen & Heidelberger, 1966; Kalus, 1972), there have been, until recently, few reports of attempts to culture non-neoplastic mucosa for more than a day or two. Johansen (1970), however, maintained such tissue for 90 hours and found significant degeneration in the later stages of culture.

Hodges & Melcher (1976) described a supplemented medium, based on Waymouth MB752/1, designed to prolong the survival *in vitro* of specimens containing both epithelial and connective tissue elements. These workers postulated that normal collagen metabolism is an essential requirement for successful long term maintenance of such tissues. They found that incorporation of ascorbic acid, ferrous sulphate and hydrocortisone into the medium enhanced the preservation of fetal mouse mandible. Although the precise role of hydrocortisone in collagen metabolism is uncertain, its ability to inhibit the release of lysosomal enzymes may help to reduce tissue damage. Defries & Franks (1977) successfully used this medium, with the addition of fetal calf serum, to culture rings of adult mouse colon for several months. The tissue adapted well to the culture system, assuming a stable form slightly different from that *in vivo*.

Using a different culture system Autrup *et al.* (1978) succeeded in maintaining human colonic mucosa for up to 20 days. These workers obtained good preservation of the mucosa during the first 48 hours, but this was followed by slowly progressive changes in the crypts: the amount of cytoplasmic mucin in the crypt epithelium diminished, with loss of goblet cell structure, and eventually disappeared; the crypts became shorter and the surviving epithelial cells assumed a cuboidal or pseudo-squamous appearance. Despite these changes, the capability for DNA and protein synthesis was retained even in the later stages of culture. Later modifications to this system (Autrup, 1980) have extended the period during which near normal structure is maintained for up to fourteen days.

We have used the organ culture system of Hodges & Melcher (1976) to investigate in detail the sequence of morphological changes occurring in adult human colonic

mucosa maintained *in vitro* for up to fourteen days. Multiple samples have been taken from each specimen of colon and the histological changes have been monitored daily. In our system, the crypts underwent profound degenerative changes in the early stages of culture, but we wish to draw attention to the remarkable regenerative changes which ensued. These changes suggest that the control and maintenance of normal colonic mucosal structure is innate rather than extrinsic.

MATERIALS AND METHODS

Samples of human colonic and rectal mucosa were obtained from surgical resections of colorectal cancer. From an apparently unaffected area remote from the tumour, a specimen of mucosa was dissected free with fine iris scissors. A sheet of tissue so obtained was 1 mm thick or less and therefore ideal for organ culture. The sheet of mucosa was then gently spread on a cork board and explants of 2×1 mm were cut with a razor blade. These explants were transferred, luminal surface uppermost, to the support phase which consisted of either 3·0 μm pore size cellulose acetate filters (Millipore) or 0·2 μm polycarbonate membranes (Nucleopore). The cultures were incubated at 37 °C at the gas-medium interface.

Initially, Trowell jars were used (Paul 1970), but subsequent cultures were set up in sterile, disposable, vented plastic petri dishes (Sterilin). The cellulose acetate filters were supported on wire grids or squares of surgical Gelfoam sponge (Upjohn) hydrated with medium; the polycarbonate membranes floated unsupported, bearing up to four explants.

The medium was that of Hodges & Melcher (1976) consisting of: Waymouth MB752/1 (Gibco); 300 μg/ml ascorbic acid (Sigma), 3·0 μg/ml hydrocortisone-21-sodium succinate (Sigma) and 0·45 μg/ml ferrous sulphate (BDH) with the addition of 100 units/ml penicillin-streptomycin solution (Gibco), 100 units/ml mycostatin suspension (Gibco) and 10–15% fetal calf serum (Gibco). The medium was changed after 24 hours and thereafter 48 hourly. The gas phase, consisting of 95% O_2 and 5% CO_2, was renewed every 24 hours.

To detect DNA synthesis, the explants were incubated under the same conditions in an identical medium, to which 6 μCi/ml tritiated thymidine (The Radiochemical Centre) had been added, for one hour prior to fixation in buffered formol-saline. The explants were embedded in paraffin wax and sectioned vertically at a thickness of 3 μm; sections were stained with haematoxylin and eosin or with PAS following amylase digestion; or were submitted to autoradiography using the dipping technique with K5 nuclear emulsion (Ilford). For the latter procedure, the slides were developed after two weeks' exposure at room temperature and then stained with haematoxylin and lightly counterstained with eosin.

The gut contains a wide variety of micro-organisms whose growth in the culture medium must be inhibited, by added antibiotics, without inflicting damage on the specimen. In an attempt to reduce the frequency of contamination of cultures by growth of gut bacteria, a series of eight specimens was first incubated at room temperature for 5 to 10 minutes in a medium containing 500 units/ml of penicillin-streptomycin solution (Gibco) and 500 units/ml of mycostatin suspension (Gibco).

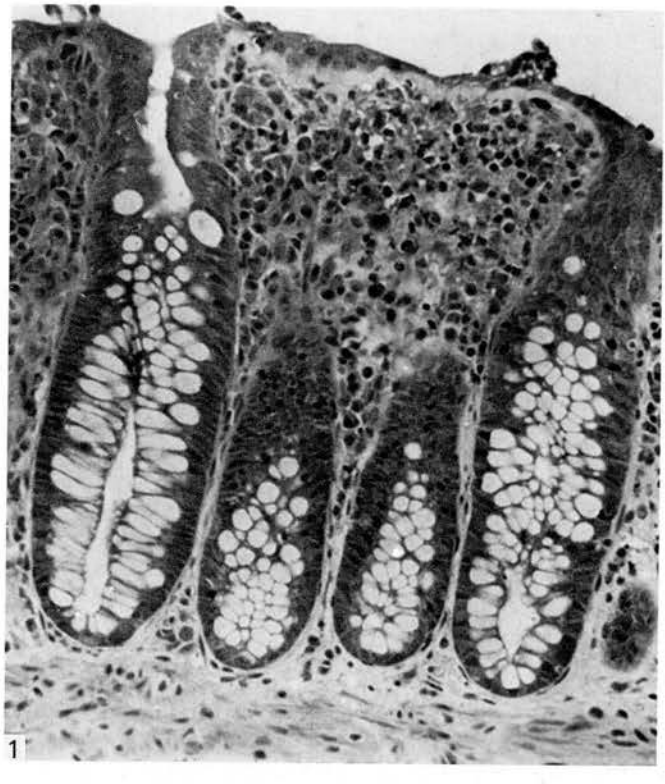

Fig. 1. A typical sample of adult colorectal mucosa prepared for organ culture. Straight, perpendicular, unbranched crypts lie close together and extend downwards to the muscularis mucosae which forms the base of the explant. The lining epithelium consists mainly of goblet cells although, in this instance, simple columnar cells predominate at the upper end; the single layer of surface epithelial cells is derived from the upward migrating crypt cells. H & E. × 200.

RESULTS

Nineteen surgical resection specimens were used as a source of explants. The explants from seven of these specimens became contaminated by gross bacterial overgrowth within the first 48 hours, in two instances despite pre-treatment with high doses of antibiotics. Of the twelve remaining series of explants which were free from obvious contamination, five nevertheless showed significant degeneration within the first 48 hours and became denuded of epithelial cells within 72 hours; three of these five had been pre-treated with high doses of antibiotics. In the remaining seven explant series, it was possible to observe the sequence of changes for up to fourteen days. Four explants per series were sampled at 24 or 48 hourly intervals.

Throughout the period of culture the explants were covered by a thick mantle of mucus within which the increasing number of necrotic cells observed during the first 72 hours suggested that migration and shedding of crypt cells were continuing. The normal histological structure of the mucosa (Fig. 1) was well preserved during the first 48 hours though some progressive depletion of cytoplasmic mucin from crypt cells was evident (Fig. 2). Thereafter, the chronology of the changes varied considerably between explants from different specimens and even from the same specimen. After 48 hours, the number of crypt cells decreased, with those in the

Fig. 2. After 48 hours in culture, the crypt cells show extensive depletion of cytoplasmic mucin and goblet cells are sparse. Mitotic activity (arrowed) can be seen in the basal two thirds of the crypts. In the surface layer of mucin, shed epithelial cells with pyknotic nuclei can be seen. H & E. ×200.

upper third of the crypt becoming flattened along the crypt axis (Fig. 3). In some cases these uppermost cells were apparently lost, leaving discrete acini or solid cell clusters in the lamina propria; these seemed to be discontinuous with the surface epithelial layer (Fig. 4) although it was possible that the pericryptal fibrocellular sheath persisted. This change occurred as early as 72 hours and by 96 hours most explants had reached this stage. Explants from two specimens, however, did not show this degree of crypt degeneration, the changes proceeding no further than those illustrated in Figure 3. During the period from about 48 hours to the time of maximum crypt degeneration between 72 and 96 hours, cell proliferation, as reflected by mitotic activity and the uptake of tritiated thymidine, was considerably depressed, but appeared not to cease completely. After the crypts had degenerated to the stages shown in Figures 3 and 4, the remnants displayed intense proliferative activity, mitotic figures and tritiated thymidine-labelled cells being evident throughout the explant; this led to progressive repopulation of the crypts between 120 and 144 hours (Fig. 5). These newly formed cells produced a monolayer epithelial lining to

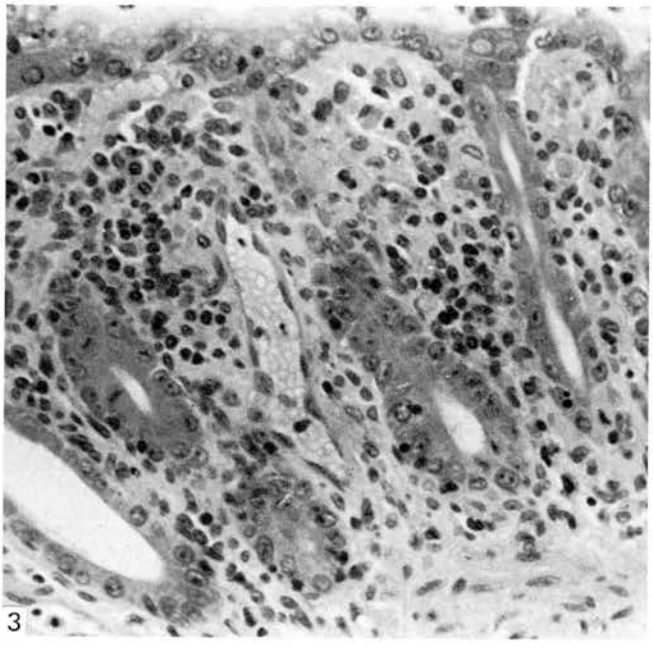

Fig. 3. After 72 hours in culture, crypts are shortened, the number of cells per crypt is reduced and the normal columnar form is lost although mitoses are still evident. Towards the upper end of the crypts, the lining cells become elongated along the plane of the crypt axis. H & E. × 300.

the reconstituted crypts, possibly guided to the surface by the persisting pericryptal fibrocellular sheaths. By 186 hours, the crypts were lined by apparently well-differentiated columnar epithelial cells (Fig. 6), but, as observed after 48 hours of culture (Fig. 2), there was little intracytoplasmic mucin and few goblet cells were apparent. At this time cell proliferation was still proceeding throughout the explant, with heavy labelling of crypt cells and numerous mitoses in the basal two thirds of the crypts. In four cultures successfully maintained beyond 186 hours, mucin-filled goblet cells reappeared after about 264 hours (Fig. 7) and this state persisted until the cultures were terminated at 336 hours.

DISCUSSION

It is not clear why some specimens maintained free from obvious contamination nevertheless failed to survive in organ culture, although the pre-treatment of three of them with high doses of antibiotics may have had a direct toxic effect. In the course of the surgical procedure, there is a variable 'warm ischaemic time' prior to the transfer of the specimen to ice cold balanced salt solution; during this period, irreversible damage may be inflicted on the mucosa in some instances.

Even in those cases where the explants were destined to survive, there was evidently a temporary breakdown of cell proliferation accompanied by profound morphological changes in the crypts. These observed mucosal changes provide an interesting contrast with the experiences of Autrup and his co-workers (1978, 1980). Originally (Autrup *et al.* 1978) these workers used CMRL 1066 medium supplemented with fetal bovine serum, glucose, hydrocortisone, β retinyl acetate, methionine, glutamine and Tricine buffer. With this system they observed progressive degeneration of the

Fig. 4. In this explant (also cultured for 72 hours) degeneration has progressed further. All that appears to remain of the crypts are isolated tubular formations or solid cell clusters whose continuity with the surface epithelium has been lost. The covering layer of mucus now contains large numbers of necrotic cells. H & E. ×250.

tissue after a few days with gross architectural and cytological changes. Modification of this system (Autrup, 1980), involving the addition of dexamethasone, pentagastrin and dimethyl sulphoxide, totally changed the behaviour of the tissue, giving excellent tissue preservation, apparently without any observed sequence of adaptive changes.

In the present study, which involves the use of a much simpler culture system, we have observed a pattern of degenerative changes similar to that described by Autrup and co-workers (1978); but following on from this there is a phase of remarkable regenerative activity, during which the regenerating crypt cells exhibit intense proliferative activity. Subsequently, they undergo a process of differentiation into apparently morphologically normal columnar epithelial cells and, in the later stages, even mature goblet cells reappear.

Despite the degenerative changes occurring in the crypts, cells continue to migrate and to be shed from the surface even when this leads to denudation of the upper ends of the crypts; evidently migration is not solely determined by 'population pressure' pushing maturing cells out of the crypts. It is also noteworthy that the surface epithelial layer of the mucosa retains its integrity at all times in successfully cultured specimens.

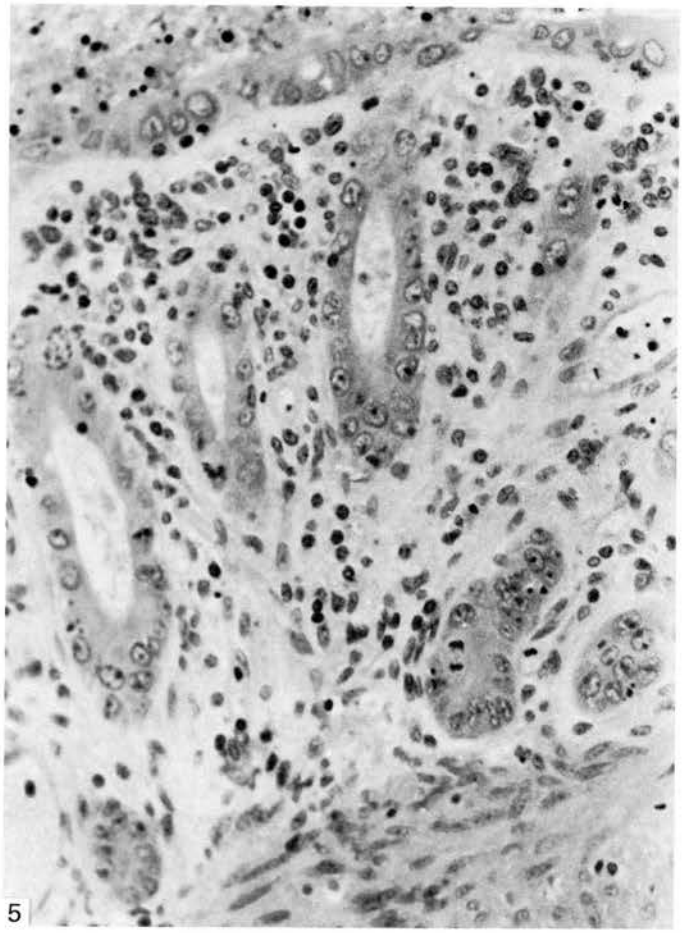

Fig. 5. After 120 hours in culture, intense proliferative activity is seen in the crypts, leading to restoration of continuity with the surface. At this stage, the crypt epithelium bears a close resemblance to that illustrated in Fig. 3 and there are no goblet cells. H & E. ×300.

Since the regenerating epithelium reproduces crypts of a similar morphological appearance to those of normal mucosa, there must be intrinsic regulatory factors within the tissue which determine the direction of cell migration and the production of new crypts lined by a monolayer of epithelial cells. Perhaps the persistence of the pericryptal connective tissue sheath is of importance in this respect. The cells lining the regenerated crypts assume the morphological characteristics of columnar epithelial cells with basally situated nuclei. This, together with the later appearance of goblet cells, suggests that the control of cell differentiation is also intrinsic. Thus, although various hormones (Johnsen *et al.* 1974; Chang & Hoff, 1980; Sharp *et al.* 1980), bile acids (Deschner & Raicht, 1979), cyclic nucleotides (Tutton & Barkla, 1980), the bulk and composition of the diet (Dowling & Booth, 1967; Levine, Steiger & Deren, 1974; Lehnert, 1979) and the activity of the autonomic nervous system (Lachat & Goncalves, 1978) have all been shown to affect cell proliferation in the gut of intact animals to a greater or lesser degree, our findings suggest that these may simply be modulators of a sophisticated local control mechanism.

Outgrowth of epithelial cells and connective tissue from the explants was not

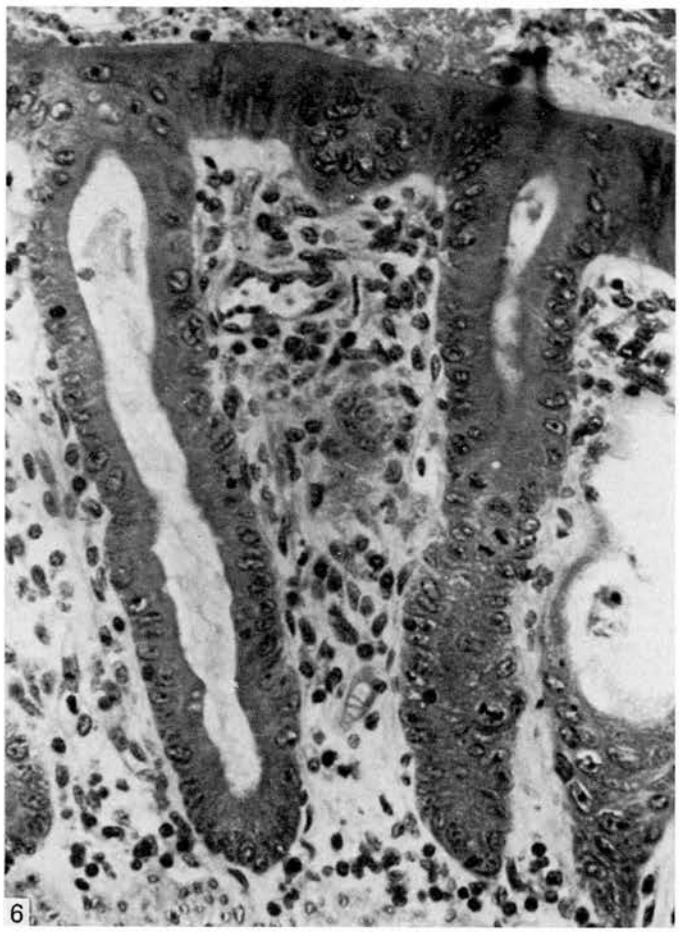

Fig. 6. Regenerated crypts after 186 hours in culture. The crypts are lined by well formed columnar cells with basally situated nuclei, but there are no goblet cells. Mitotic figures can be seen and the appearance generally resembles that found after 48 hours in culture (cf. Fig. 2). H & E. ×350.

observed. In common with Autrup (1980), we found that the type of support phase used affected the behaviour of the explant in culture. With polycarbonate membranes, a single cell layer of epithelium covered the underside of the explant in the later stages of culture, whereas this effect was never observed with cellulose acetate filters. This phenomenon, which did not appear to affect survival, seems to be related to the intimacy of contact between the explants and the support.

The residual epithelial formations shown in Figure 3 are capable of intense proliferative activity and may comprise the functional stem cell compartment, possibly augmented by potential stem cells (Withers, 1976; Wright, 1978).

Thus, it appears that long term culture of human colonic mucosa is achievable under the correct culture conditions, and that the behaviour of the tissue *in vitro* can be manipulated by varying the composition of the medium. It has yet to be evaluated how long the regenerated mucosa will survive and whether a steady state between cell proliferation and cell loss can be achieved in the regenerated crypts. Clearly, such a system will have a useful role in the study of mucosal function both in normal and diseased mucosae, and in evaluating the tissue response to carcinogens,

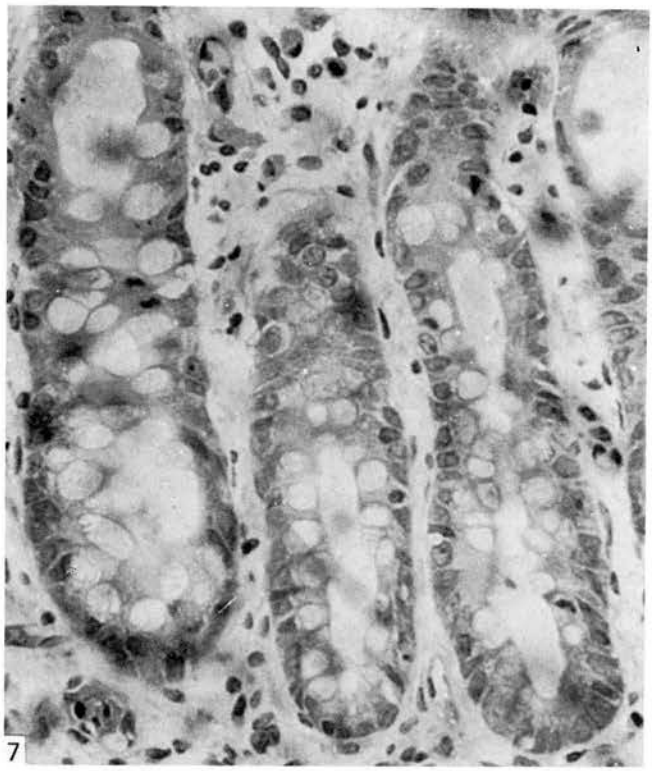

Fig. 7. After 264 hours in culture, well-formed goblet cells are again seen and the appearances are virtually indistinguishable from the preculture mucosa (cf. Fig. 1). H & E. ×350.

drugs and presumed trophic factors. A considerable advantage of the *in vitro* approach is that it isolates the tissue from systemic influences which render difficult the interpretation of results obtained *in vivo*.

SUMMARY

Using a system designed to preserve, *in vitro*, both the epithelial and the connective tissue elements, we have maintained adult colonic mucosa in organ culture for up to 336 hours and have investigated the sequential morphological changes which occur. During the first 48 hours, normal micro-architecture is preserved, but there is progressive loss of cytoplasmic mucin from crypt cells. Subsequently, accelerated degenerative changes develop; cells are lost from the crypts and, because cell proliferation in the crypt is reduced, these lost cells are not replaced.

For a time, the crypts are represented by discrete acinar formations or clusters of cells in the lamina propria, apparently discontinuous with the intact surface epithelial layer. These remnants manifest intense proliferative activity during the period between 72 and 96 hours after explantation, leading to the restoration of well formed crypts lined by columnar epithelial cells between 120 and 144 hours; differentiation of goblet cells ensues and this state of virtually normal structure persists until the termination of culture between 186 and 336 hours.

It is concluded that the determination of normal crypt structure and of crypt-cell differentiation is governed by intrinsic control mechanisms although these may be

subject to extrinsic modulation. Whether or not the degeneration phase can be eliminated, it is clear that long term culture of adult human colonic mucosa is possible. Such a system may be useful in the study of mucosal function and of mucosal response to drugs, carcinogens and trophic factors.

This work was supported by a grant from the North of England Council of the Cancer Campaign for Research. Mr C. J. Pritchett is in receipt of a Prophit Scholarship from the Royal College of Surgeons. We are grateful to Mrs E. Wallace and Mrs S. Robinson for technical assistance, to Miss E. Wark and Miss Y. Allen for typing the manuscript, and to the Surgeons of the Royal Victoria Infirmary and Newcastle General Hospital for providing material.

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Human colorectal tumours in short-term organ culture

A stathmokinetic study

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(*Received* 5 *September* 1981; *revision accepted* 26 *February* 1982)

Abstract. Short-term organ culture, using a technique to preserve epithelial/stromal interaction and metabolism, is a useful technique for carrying out kinetic studies on human colorectal carcinoma and adjacent normal mucosa, providing initial perturbations of proliferative indices are allowed to settle. Tumours require 3·0 μg/ml vincristine for complete metaphase arrest compared with mucosa, which needs 0·5 μg/ml, a 6-fold difference. Using a stathmokinetic technique, the birth rate of tumour cells is 10·21 cells/1000 cells per hr, compared with 7·73 cells/1000 cells per hr for mucosa, a statistically significant difference ($P < 0·01$).

It is well known from clinical observation that an unresected rectal carcinoma may take several years to grow large enough to cause symptoms. Serial barium studies on colorectal tumours showed a mean tumour volume doubling time of 620 days (Welin, Youker & Spratt, 1963). What little data exist from *in vivo* kinetic studies suggest that tumour cells cycle more slowly than normal epithelial mucosal cells in the large bowel (Camplejohn, Bone & Aherne, 1973; Hoffman & Post, 1967).

The literature on cell proliferation in the solid tumours most common in man is relatively small, mainly due to the enormous practical difficulties of carrying out *in vivo* studies on gravely ill patients. One important problem has been obtaining the large number of samples which are required for accurate and representative studies. Data from a few biopsies from each of many patients can be amalgamated (Camplejohn *et al.*, 1973; Refsum & Berdal, 1967; Aherne & Buck, 1971), but this gives no information about the proliferative status of the individual tumours, and the heterogeneity between tumours, as well as within them (Aherne *et al.*, 1977) cannot be ignored. Wright and his colleagues (Wright *et al.*, 1977) attempted to obtain proliferative rates from individual gastric carcinomas using an *in vivo* stathmokinetic technique, but because the resulting confidence limits were so wide, the results were of uncertain value. In addition there are other constraints of an ethical nature on *in vivo* studies when tritiated thymidine and metaphase arresting agents need to be administered.

Because of these problems, the concept of organ culture as a model for cell kinetic studies is attractive and has been used for some time (Steel & Bensted, 1965; Eastwood & Trier, 1973; Lieb & Lisco, 1966; Bleiberg & Galand, 1976; Lescher, Schiffer & Phanse, 1977). Organ culture avoids the mesenchymal overgrowth known to occur in single cell systems, and

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maintains the anatomical inter-relationships of cells. Over many years there have been reports of colorectal tumours maintained in culture for long periods (Röller, Owen & Heidelberger, 1966; Kalus, 1972; Wolff, Smith & Wolff, 1972) but it is only recently that techniques have become refined enough to support normal mucosa for more than a few days (Autrup, 1980; Senior *et al.*, 1982).

Despite these advantages several problems have limited the use of this type of system in kinetic studies. One troublesome occurrence is that of 'edge labelling' seen early in culture, when DNA synthesis, as evidenced by thymidine labelling, is observed only in the peripheral 150 μm of explanted tissue fragments. It has been suggested that this phenomenon may be caused by an oxygen diffusion defect (Steel & Bensted, 1965), but an additional explanation could be stimulation of cell proliferation at the site of trauma (Bullough, 1965). Mitotic activity appears depressed during the first few hours of culture (Fabrikant, Wisseman & Vitak, 1969; Simnett & Fisher, 1973) and it is notable that previous *in vitro* work on cell proliferation has been carried out largely during this early period of culture. The usual practice has been to select areas of high labelling density at a time when cell proliferation in the cultured specimen is obviously seriously perturbed. We have attempted to avoid these problems by carrying out our kinetic studies after 18 hr in culture, when these perturbations appear to have settled and labelled nuclei and mitoses are uniformly distributed throughout the tissue (Pritchett *et al.*, 1981).

Our organ culture method is based on that of Hodges & Melcher (1976) and is designed to support tissues containing a mixture of epithelial and connective tissue elements (Defries & Franks, 1977).

Cell proliferation was investigated by the technique of metaphase arrest using vincristine. Clear advantages of an *in vitro* system for this technique are firstly, that dose-response experiments for the particular stathmokinetic agent can be carried out, and secondly, that data can be obtained from large numbers of tissue samples, thus ensuring the accurate identification of the duration of linear accumulation of metaphases, and so avoiding any initial lag phase and the final plateau phase when metaphases are degenerating (Wright & Appleton, 1980). The stathmokinetic technique also has distinct advantages over autoradiographic techniques in that it is simpler, less time consuming, less expensive and less laborious. If precautions are taken, cell birth rates obtained by metaphase arrest are reasonably precise (see Discussion). However, it should be emphasized that without exhaustive morphological and functional tests, it is not known whether tissue in organ culture is behaving as *in vivo*, since it is removed from a large number of hormonal, neurological and nutritional influences and is exposed to a relatively crude culture 'milieu'.

MATERIAL AND METHODS

Ten adenocarcinomas from descending colon, sigmoid colon or rectum were obtained from patients undergoing surgical resection. The specimens were taken directly from the operating theatre and were rapidly cleaned, cooled and transported to the laboratory in ice-cold buffered Hanks' solution (Gibco). Normal-looking mucosa, as far removed from the tumour as possible (an average of 14 cm distant), was gently stripped from the submucosa. Multiple pieces measuring approximately 1·5 mm square were cut with a razor blade and placed, luminal surface uppermost, on micropore filters (Millipore Type SS 3·0 μM). Explants of similar size were obtained from the growing edge of the tumour within the bowel lumen. The filters were placed on stainless steel grids in silica petri dishes and housed in Trowell jars (Trowell, 1959). The culture medium (Hodges & Melcher, 1976) consisted of Waymouth MB

752 medium (Gibco) containing 10–15% foetal calf serum and was supplemented with 300 μg/ml ascorbid acid (Sigma), 3·0 μg/ml hydrocortisone-21-sodium succinate (Sigma), 0·45 μg/ml ferrous sulphate (B.D.H.), 100 units/ml penicillin per streptomycin solution (Gibco) and 100 units/ml mycostatin suspension (Gibco). The pH of the medium was checked and adjusted before filter-sterilization (Millipore Millex). The gas phase consisted of 95% O_2 and 5% CO_2 and the tissue explants were carefully maintained at the fluid-gas interface.

Explants were cultured for 18 hr at 38°C and then the stathmokinetic experiments were commenced. Two dose-response experiments were carried out using doses of vincristine (Oncovin, Lilly) ranging from 0·1 μg/ml to 4 μg/ml made up in fresh, buffered, warmed medium. Eight more stathmokinetic experiments were carried out using the minimum doses of vincristine required for complete metaphase arrest, namely 0·5 μg/ml for mucosa and 3·0 μg/ml for tumour. Specimens were fixed in neutral buffered formal saline from time zero up to a maximum of 6 hr post-vincristine, using time intervals of 20–40 min. The material was processed to paraffin wax; serial sections measuring 4 μmm thickness were prepared and stained with haematoxylin and eosin. At least two tissue specimens were obtained per time point and two counts of a minimum of 2000 crypt cells were obtained per tissue sample. All stages of mitosis were counted, and evidence of metaphase escape in the later parts of the experiments was carefully sought. Since it was not possible to orientate mucosal crypts in perfect longitudinal section as the specimens were so small, all crypt cells were counted whatever their orientation. All the data used for the calculation of cell birth rates were obtained between 1 and 4 hr when arrest was effective and degenerating metaphases were not seen.

The results were plotted as mitotic index (I_m as a percentage) against time and lines were fitted to the data using least squares and assuming a rectangular age distribution. The cell birth rate was calculated from the regression line and was expressed as cells per 1000 cells per hr.

RESULTS

The first dose-response experiment was carried out over 180 min using doses of 0·1, 0·5, 1·0 and 1·5 μg/ml vincristine (Figs 1 and 2). All doses produced typical arrested metaphases in

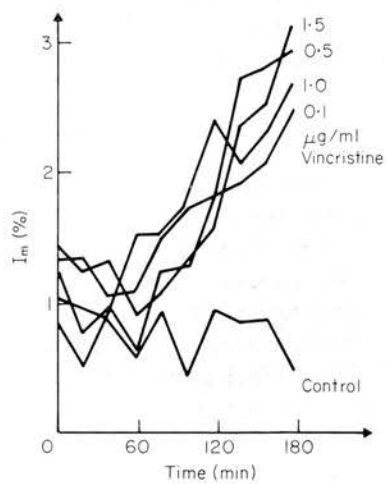

Fig. 1. Case 1. Vincristine dose-response experiment. Mucosa.

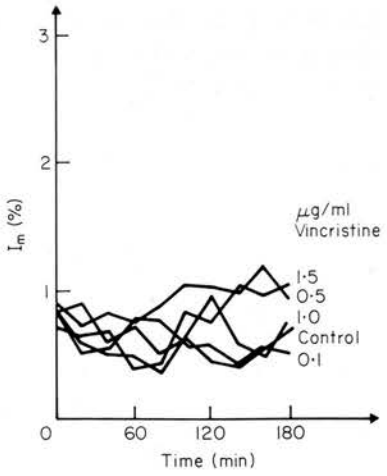

Fig. 2. Case 1. Vincristine dose-response experiment. Tumour.

mucosa and tumour; in mucosa the minimum dose required for complete metaphase arrest was 0·5 μg/ml, but in tumour post-mitotic figures were clearly evident at 180 min even at the highest dose.

A second dose-response experiment (Figs 3 and 4) extended the time range and focused on higher doses for tumour (1·0, 2·0, 3·0 and 4·0 μg/ml) and lower doses for mucosa (0·2

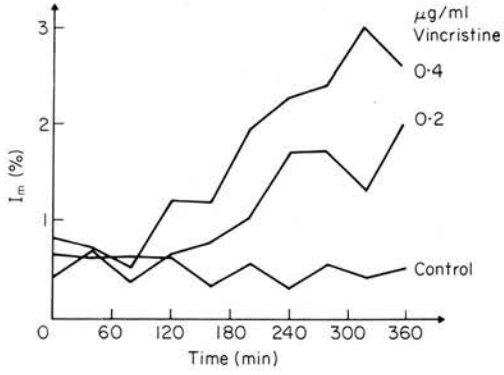

Fig. 3. Case 2. Vincristine dose-response experiment. Mucosa.

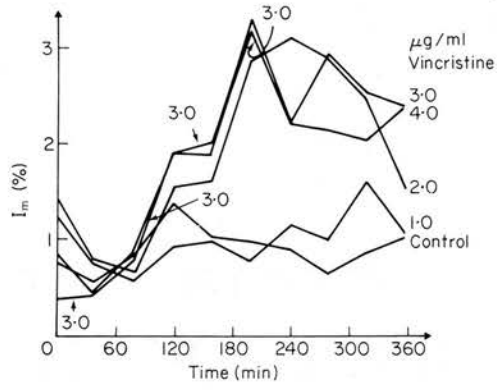

Fig. 4. Case 2. Vincristine dose-response experiment. Tumour.

and 0·4 μg/ml). The 0·4 μg/ml dose for mucosa showed accumulation but two anaphases were seen late in the experiment. The minimum dose for complete arrest in mucosa was therefore taken to be 0·5 μg/ml. The three highest doses, 2·0, 3·0, and 4·0 μg/ml, in the

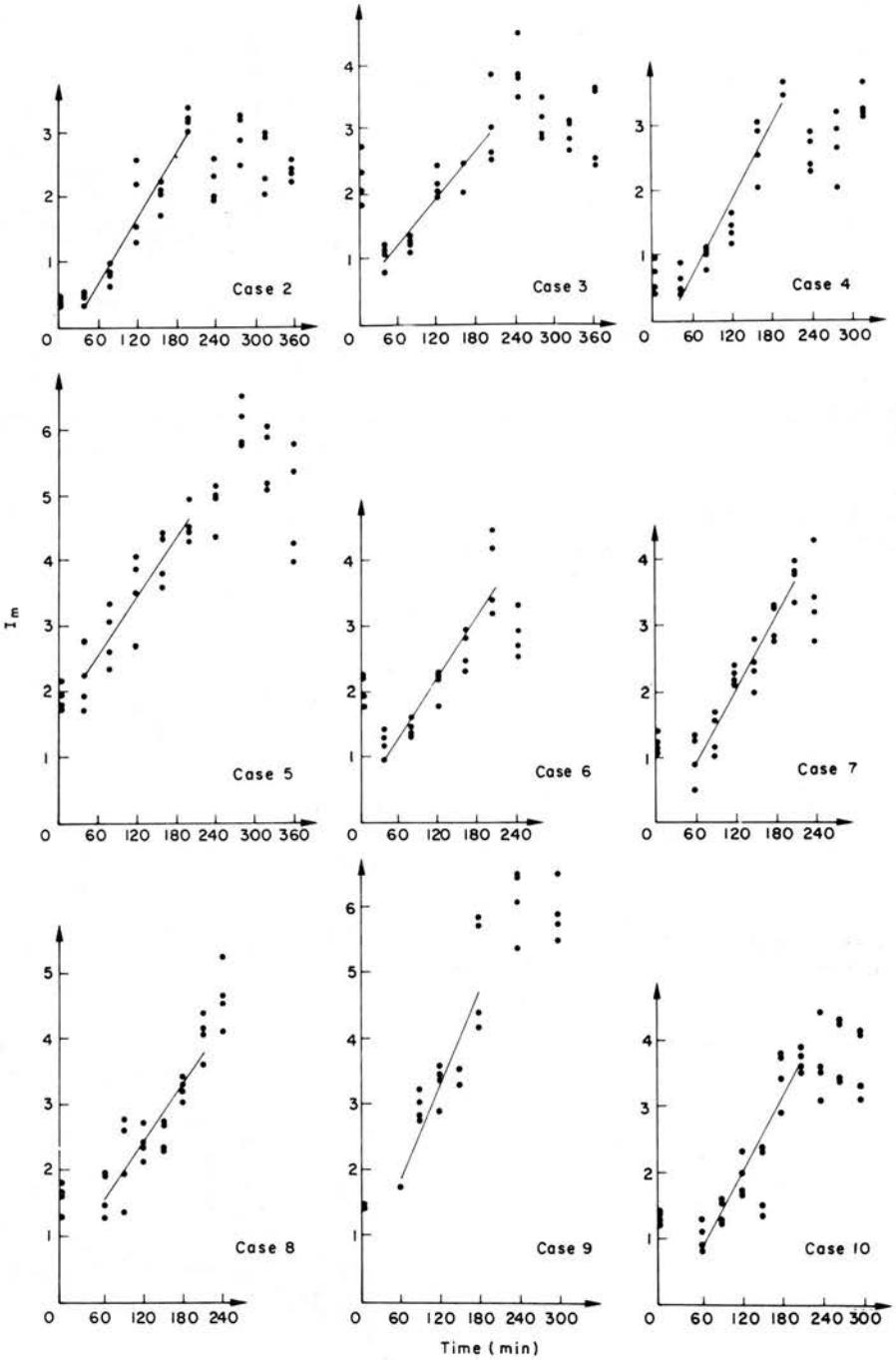

Fig. 5. Metaphase accumulation in mucosa of nine cases using 0·5 μg/ml of vincristine. Two specimens per time point with two counts of >2000 cells per specimen. The lines are fitted assuming a rectangular age distribution.

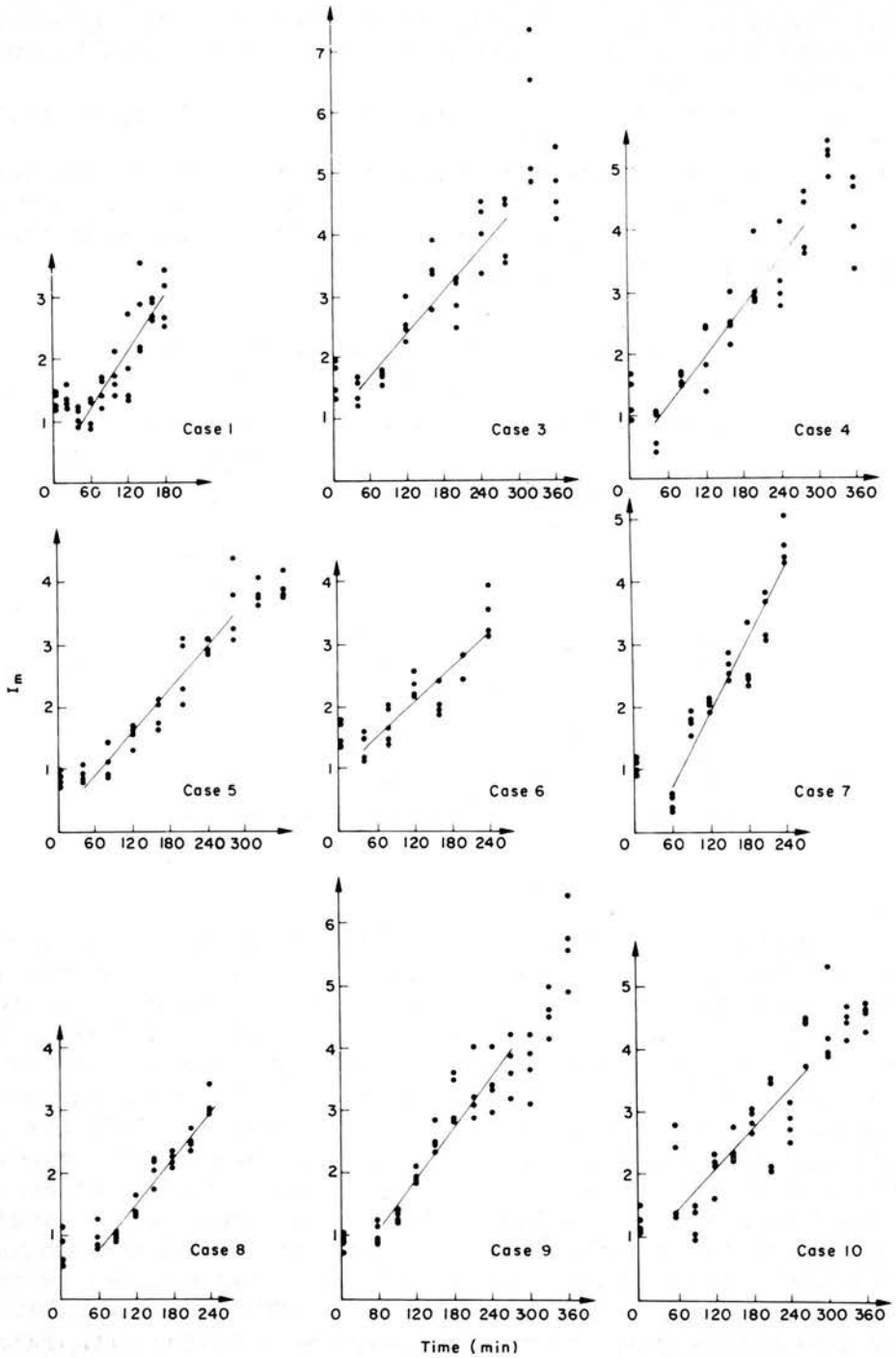

Fig. 6. Metaphase accumulation in tumour of nine cases using 3·0 μg/ml vincristine. Two specimens per time point with two counts of >2000 cells per specimen. The lines are fitted assuming a rectangular age distribution.

tumours caused metaphase accumulation and the 3·0 μg/ml dose was the lowest at which no evidence of metaphase escape was apparent. These two dose-response experiments therefore demonstrated a six-fold difference in the minimum dose of vincristine required for complete arrest in mucosa and tumour.

The eight further experiments using 0·5 μg/ml for mucosa and 3·0 μg/ml for tumour yielded the results shown in Figs 5 and 6.

Estimates of the individual birth rates appear in Table 1. Means of the birth rates for each series of nine experiments were 7·7 cells/1000 cells per hr for mucosa and 10·2 cells/1000 cells per hr for tumour. These birth rates are significantly different using a paired *t* test ($P < 0·01$) bearing in mind that experiments 1 and 2 are mismatched.

Table 1. Cell birth rates for mucosa and tumour expressed as cells/1000 cells per hr. The means are significantly different using a paired *t* test ($P < 0·01$)

Mucosa		Tumour	
Case	Birth rate	Case	Birth rate
1	9·15	2	10·08
3	6·86	3	7·36
4	7·87	4	11·33
5	6·89	5	9·00
6	5·47	6	9·50
7	11·60	7	10·89
8	7·26	8	8·62
9	8·12	9	14·00
10	6·36	10	11·09
Mean	7·73	Mean	10·21

DISCUSSION

We have demonstrated a difference between mucosa and tumour in sensitivity to the metaphase arresting properties of vincristine. It has proved a considerable advantage to be able to carry out dose-response experiments in this *in vitro* system because there is evidence that vincristine has different effects at different doses (Marsden, 1972; Creasey, 1975). Conversely, the same effect may require different doses in different tissues within the same animal (Tannock, 1967; Nome, 1975). Indeed, different parts of a single tumour can react differently to cytotoxic drugs (Hakansson & Tropé, 1974; Heppner *et al.*, 1978). This system allows for multiple sampling of tumours although we used only two biopsies per time point.

Vincristine has previously been shown to be a satisfactory metaphase arresting agent (Tannock, 1967; Smith, Thomas & Riches 1974) giving linear accumulation of metaphases for longer than colcemid or vinblastine. However, it is important to use the minimum dose for complete arrest, because not only does increasing dose appear to increase the rate of degeneration of metaphase (Smith *et al.*, 1974; Clarke, 1971), but also high doses may depress DNA synthesis, giving lower mitotic counts by reducing flux through G_2 (Fitzgerald & Brehaut, 1970). Data for use in calculating cell birth rates were obtained only from the linear sections of the metaphase accumulation graphs, and, though degenerating metaphases were seen, their presence was only noticeable after 4 hr in both tumour and mucosa. In

tumours, however, the plateau phase commenced earlier than in mucosa, at about 200 min, possibly representing a toxic effect, at the higher dose of vincristine, on cells in G_2 or in the late S phase. Jellinghaus, Schultze & Maurer (1977) showed that flux into or out of DNA synthesis was not affected by standard doses of vincristine in the mouse jejunum. It should be noted that in our system, higher than optimum doses of vincristine produced no alteration in rates of metaphase accumulation. All our experiments had a lag period of around 60 min and although it has been suggested that the duration of this period is dose-related (Taylor, 1965) we found no evidence to support this. The distribution of arrested metaphases across the explant was uniform as shown previously for normal mitoses after 18 hr in culture (Pritchett *et al.*, 1981).

Although it is not known precisely how vincristine exerts its anti-tumour action in human patients (Camplejohn, 1980; Klein, 1980), it is relevant to ask if the difference in sensitivity (to vincristine) between colorectal tumours and mucosa *in vitro*, reflects, in any way, the depressing lack of efficacy of chemotherapy with this agent in the treatment of large bowel cancer. Camplejohn *et al.* (1973) hinted that there was a dose-response difference in their *in vivo* study. They noted 'only very rare and usually abnormal telophases in the tumour' using the standard 0·045 mg/kg dose. This suggests incomplete block and no such figures were seen in the present study using 3·0 μg/ml. It is difficult to relate *in vitro* doses to the pharmacodynamics of the drug *in vivo* (Dickson & Suzanger, 1976), but 3·0 μg/ml is at least sixty times higher than the maximum permitted clinical dose. It is hard to explain the difference in response by tumour and mucosa except on the basis of a cellular resistance, since the factor of poor blood supply to the tumour has been eliminated.

The calculated cell birth rates in colorectal tumours in this study are significantly higher than in the adjacent apparently normal mucosa. The mean cell birth rate for tumours is 10·2 cells/1000 cells per hr twice as high as the value obtained by Camplejohn *et al.* (1973). Our estimate for normal mucosa is, however, considerably lower, and this is worrying because the inherent errors of the stathmokinetic technique all lead to underestimation of cell birth rates, suggesting that there may be a deficiency in this *in vitro* system for mucosa. It could also be argued that tumour cells are cycling faster because of improved nutrition and oxygenation in culture whereas mucosa is always maximally nourished *in vivo* and could only perform less efficiently *in vitro*. In other words, our tumour explants may have reverted to a former, faster rate of cell division, though they may simply be better adapted than mucosa to this particular culture system.

The spread of data points about the fitted line is larger for the tumours than the mucosa. This confirms the concept of kinetic heterogeneity between tumours of similar histological appearance (Fulker, Cooper & Tanaka, 1971). However, the spread of mitotic indices within a single tumour (four separate counts from two tissue samples) is no wider than that of normal mucosa. It may be possible, by the accurate determination of cell birth rates in individual tumours, to identify separate subgroups within a histologically homogenous population.

Although we have chosen to calculate cell birth rates assuming a rectangular age distribution, the value is altered little using an exponential age distribution. However, it is possible to go on to calculate the mean turnover time or potential doubling time, which in mucosa is 129 hr for a rectangular distribution and in tumours, 68 and 98 hr, for exponential and rectangular age distributions, respectively, the true value lying somewhere between.

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David Appleton

J. Anat. (1985), **141**, pp. 171–179
With 2 figures
Printed in Great Britain

Cell proliferation in human colorectal mucosa in organ culture: the early adaptive changes

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(*Accepted* 29 *November* 1984)

INTRODUCTION

The study of cell proliferation in human intestinal epithelium is highly relevant to the investigation of the normal regulatory mechanisms involved in cell renewal and adaptation, and to the development of malignancy. The classic techniques for elucidating cell cycle parameters (Steel, 1977) involve the use of tritiated thymidine (^{3}HTdR) or metaphase-arresting agents and require multiple tissue samples taken over a period of time. Such *in vivo* studies are possible (Deschner & Kipkin, 1978) but there are considerable ethical and practical advantages in using material from surgical resection specimens in an organ culture system.

Organ culture of solid human tumours has provided some kinetic data (Steel & Bensted, 1965; Fabrikant, Wisseman & Vitak, 1969), but it is the culture of normal intestinal mucosa that has proved more challenging (Trier, 1976). Although prolonged organ culture of intestinal mucosa is now possible (Autrup, 1980; Senior *et al.* 1982), kinetic data relating to normal colonic mucosa in organ culture is largely limited to estimates of epithelial mitotic index (I_m) and labelling index (I_s) from short term experiments. A major problem in the interpretation of this data is uncertainty about the short term proliferative changes, either adaptive or degenerative, which take place early in organ culture, expressed as temporal variations in I_m and I_s.

The most disturbing phenomenon has been that of 'edge labelling'. When tissue explants, normal or neoplastic, are incubated with ^{3}HTdR in order to label the cells in the S (or DNA synthetic) part of the cell cycle, only the most peripheral 100–150 μm of the tissue takes up label (Lieb & Lisco, 1966). Despite altering the conditions of culture, the dosage of ^{3}HTdR, and the length of incubation, the depth of tissue labelling does not increase in the first few hours of culture. Labelling indices have thus been derived from the peripheral rim of cultured tissue only. Several authors have hinted briefly that edge labelling might possibly be due to traumatic stimulation of proliferative activity at the site of wounding (Hayashi, Yatani, Apostol & Stemmermann, 1974; Eastwood & Trier, 1973; Steel, 1977), but most workers have incriminated anoxia of the central part of the explant as the cause of central inactivity. Although hyperbaric oxygen does increase the depth of edge labelling (Fabrikant *et al.* 1969) its effects are unphysiological and toxic (MacDougall & Coupland, 1967; Heppleston & Simmett, 1964; Haugaard, 1968).

Steel (1977) has emphasised the different rationale between short term 'flash

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labelling' *in vitro* studies where kinetic indices have not had time to change after excision from the host and may for a brief time (perhaps an hour or less) represent *in vivo* levels, and the longer term *in vitro* experiments lasting several hours where ongoing proliferative behaviour is monitored. Clearly such experiments provide more kinetic information, but whether this information is useful depends on proliferative indices being stable and similar to those *in vivo*. These aims are difficult to achieve.

In view of the difficulties relating to the determination of proliferative indices *in vitro*, it was considered necessary to follow mitotic and labelling indices through at least the first 24 hours in organ culture, using a proven organ culture technique and a tissue that could be easily orientated. The objectives were to try to define the adaptive kinetic changes in colorectal mucosa and to find a period of proliferative stability comparable to the kinetic state *in vivo*.

MATERIALS AND METHODS

Mitotic index in vivo

Mitotic index was determined in sigmoidoscopic or colonoscopic biopsies from twelve patients whose rectosigmoid mucosa was histologically normal. Immediate fixation in formalin was used to prevent the disappearance of mitotic figures, a phenomenon which has been observed when fixation is delayed (Bullough, 1950), and to ensure that the calculated I_m truly represented the *in vivo* values. A minimum of 2000 epithelial cells per specimen was counted, and the proportion in mitosis expressed as a percentage.

Organ culture technique: preparation of sections

Six resection specimens were obtained from patients undergoing surgery for carcinoma of the descending colon, sigmoid or rectum. They were taken directly from the operating theatre and were rapidly cleaned, cooled and transported to the laboratory in ice cold buffered Hank's solution (Sigma). Mucosa which was normal in appearance, as far removed from the tumour as possible, was gently stripped from the submucosa. Multiple pieces roughly 1·5 mm square were cut with a razor blade and placed, luminal surfaces uppermost, on micropore filters (Millipore, type SS 3·0 μm). The filters were placed on stainless steel grids in silica petri dishes and housed in Trowell jars (Trowell, 1959). The culture medium (Hodges & Melcher, 1976) consisted of Waymouth MB 752 medium (Gibco) containing 10–15% fetal calf serum, and supplemented with 300 μg/ml ascorbic acid (Sigma), 3·0 μg/ml hydrocortisone-21-sodium succinate (Sigma), 0·45 μg/ml ferrous sulphate (B.H.D.), 100 units/ml penicillin/streptomycin solution (Gibco) and 100 units/ml mycostatin suspension (Gibco). The pH of the medium was checked and adjusted to 7·4 before filter sterilization (Millipore Millex). The gas phase consisted of 95% oxygen and 5% carbon dioxide and the tissue explants were maintained at the fluid–gas interface. Following the kinetic experiments (see below), the explants were fixed in neutral buffered formol saline and then processed routinely into paraffin wax. They were embedded at 90° to the mucosal surface and serial sections at 3 μm were cut longitudinally through the mucosal crypts. Sections were then stained with haematoxylin and eosin or subjected to autoradiography using a dipping technique and a two weeks exposure period, then counterstained with Harris' haematoxylin.

Table 1. *Labelling index and average grain count for specimens of colorectal mucosa from two cases, measured early during organ culture, and after about one day*

Because of the low labelling index the grain count is not given for the middle of the explants at one hour.

	Case 1: labelling index			
	1 hour		20 hours	
	Edge	Middle	Edge	Middle
Explant 1	8·27	0·30	4·45	2·66
Explant 2	5·70	0·13	3·83	3·88
Grain count per nucleus	High	—	45	14

	Case 2: labelling index			
	1 hour		26 hours	
	Edge	Middle	Edge	Middle
Explant 1	9·74	0·37	4·17	3·26
Explant 2	5·94	0·00	3·39	3·14
Grain count per nucleus	High	—	54	18

Kinetic experiments

In experiments 1 and 2, dishes of explants were flash labelled after one hour in culture by the addition of tritiated thymidine (Amersham International) to the medium for a further hour in a concentration of 6 μCi/ml of medium. Samples were fixed after one hour and after 20 hours (experiment 1) and 26 hours (experiment 2). The tissues were processed and autoradiographs were prepared. Labelling index was evaluated by counting a minimum of 1500 epithelial nuclei and determining the percentage labelled, using a minimum of five grains per nucleus as the criterion for labelling. Two separate counts were made in a 0·3 mm thick zone at the edge of the explant (usually about three crypts in width) and these figures were compared with two counts made at the centre of the explant; two explants were evaluated per time point in both experiments. Autoradiographic grain counts were undertaken in twenty labelled cells at both edge and centre in both experiments and these too were compared at the two time points.

In experiments, 3, 4, 5 and 6, dishes of explants were flash labelled with ^{3}HTdR as before, at intervals up to 24 hours in culture. Labelling indices were calculated as above for the edge and centre, and in addition corresponding counts of mitotic index, each based on the evaluation of 1500 cells, were made.

RESULTS

The mean value of native mitotic index from the twelve endoscopic biopsies of rectosigmoid mucosa was 1·01 % and the standard error of the mean was 0·05 %.

Table 1 compares labelling indices at the edge of the explants with those in the centre in experiments 1 and 2 where I_s was estimated at 1 hour in culture and at 20 and 26 hours respectively. At 1 hour there were virtually no labelled cells centrally, while there was conspicuous edge labelling. At 20 and 26 hours edge labelling was reduced and the labelling index at the centre of the explant had risen to a level very

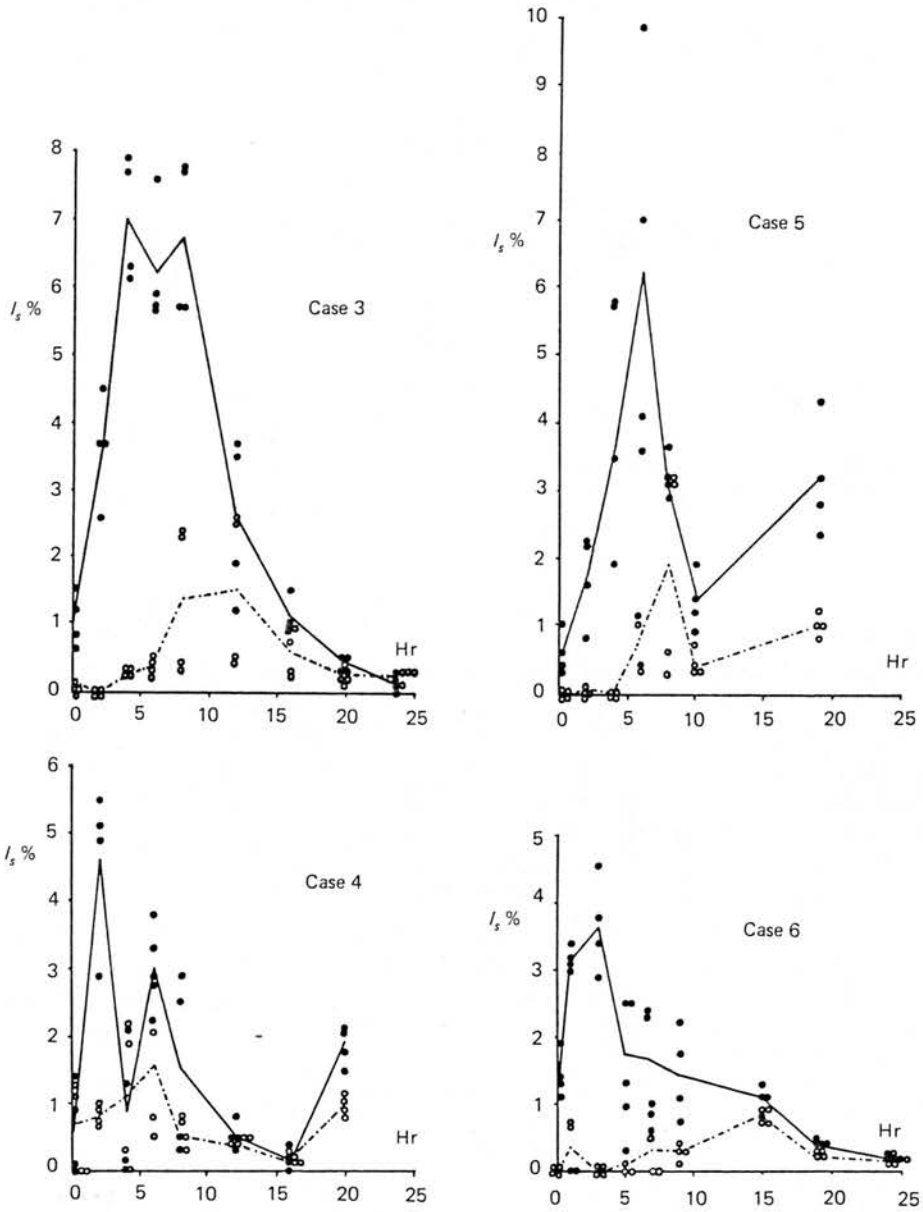

Fig. 1. Graph of labelling index (I_s) over the first 24 hours in culture, comparing the edge 0·3 mm (●—●) with the centre (○–·–○) of the explants. Each value of I_s was calculated from at least 1500 cells, and there were two explants per time point.

close to that seen at the edge. There remained, however, a marked difference in average grain counts, the cells at the periphery of the explant being much more heavily labelled than those at the centre.

These changes were further investigated by comparing I_s in the edge and centre in the material in experiments 3, 4, 5 and 6 at intervals during the first 24 hours in culture (Fig. 1). There was great variability in the results but constant features were: (1) Low values for I_s when tritiated thymidine was added to the tissue at time zero.

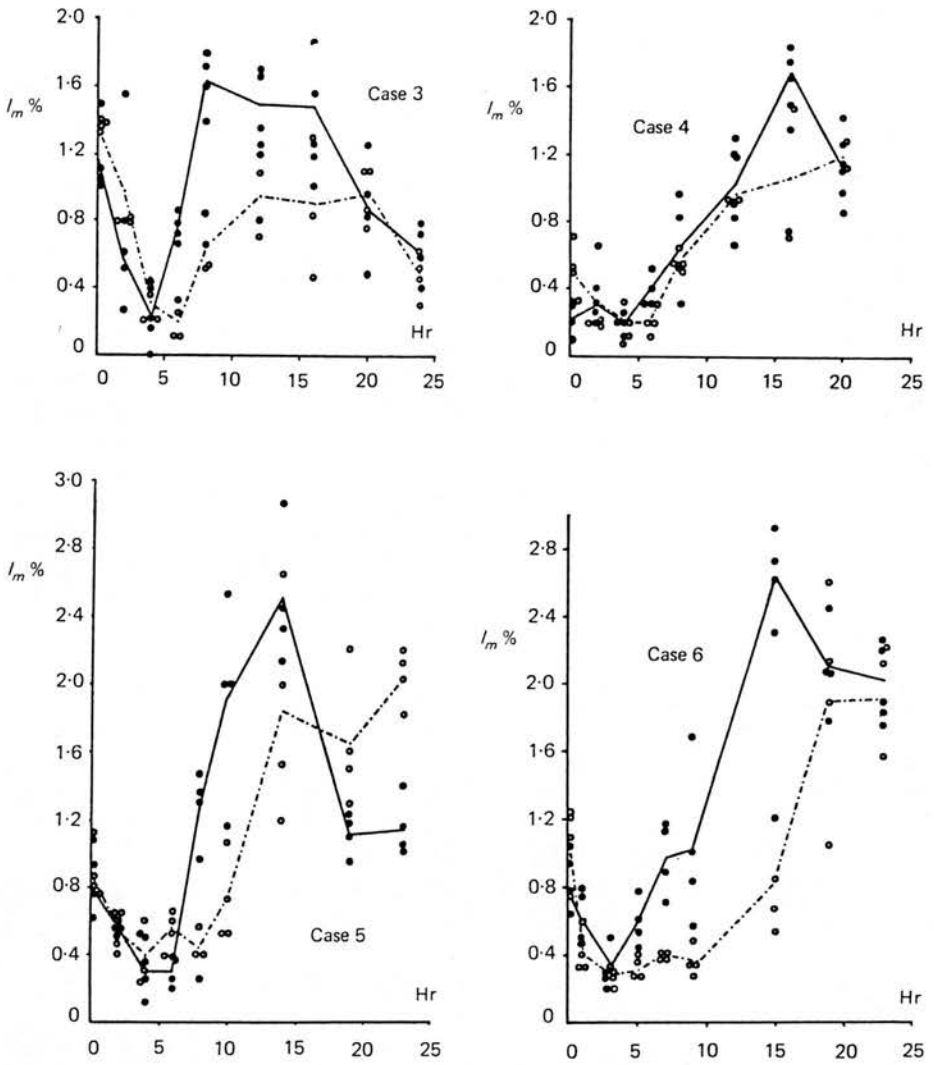

Fig. 2. Graph of mitotic index (I_m) over the first 24 hours in culture, comparing the edge 0·3 mm (●—●) with the centre (○–·–○) of the explants. Each value of I_m was calculated from at least 1500 cells, and there were two explants per time point.

(2) A peak of edge labelling at 1 to 8 hours in culture. (3) A decline of values after 8 hours with the appearance of a variable degree of central labelling.

Figure 2 illustrates plots of I_m against time in experiments 3, 4, 5 and 6. A rapid fall or an initial depression of I_m was apparent, followed by a recovery phase, becoming stable between 8 and 18 hours in culture. Mean values for I_m during this plateau phase were similar to the 'baseline' values observed in biopsy specimens. Recovery of I_m commenced at about 5 hours, first in the tissue edge, the central values catching up at about 18 hours.

The preservation of tissue after 24 hours in culture was good with no central necrosis of the explants.

DISCUSSION

It is desirable to use human tissue rather than animal models in studies designed to investigate human disease, and the use of organ culture for kinetic studies has clear advantages over cell culture, where dissociation procedures, mechanical or enzymatic, cause a large number of proliferative, structural and biochemical changes (Hodges, 1976). It is difficult, however, to measure the degree of trauma and the extent of the resulting adaptive changes in the preparation of explants for organ culture. Even with care and gentle handling, tissue has to be cut and trauma is a potent stimulus to DNA synthesis and cell division (Bullough, 1965; Schilling, 1968; Gelfant, 1962).

Clearly, therefore, the claim that cells or tissues are 'normal' in culture must be based on exhaustive structural and functional studies. It has already been shown (Senior *et al.* 1982) that mucosa in the present organ culture system is structurally well preserved up to 48 hours, and that cells are still dividing after 4 weeks.

The aim of the present work was to study the early adaptive changes of human colorectal mucosa in organ culture, for the purpose of identifying a period of proliferative stability during which kinetic experiments could be carried out using techniques that are now standard *in vivo* (Steel, 1977). The experiments have shown marked temporal fluctuations in I_m and I_s which vary between sites within the explant. The rapidity and complexity of the kinetic changes occurring after explantation are unlikely to be due solely to a failure of oxygen to diffuse to the centre of the explants, and a number of alternative factors may be of importance.

It appears that when tissue is explanted, it undergoes a metabolic shock for the first hour and even cells already apparently committed to DNA synthesis are inhibited. Only mitosis, a process resistant to the most adverse conditions, is observed and this rapidly disappears since the duration of mitotic phase is short. The wave of edge labelling appearing after one hour could represent cells, previously blocked by the trauma of explantation, resuming DNA synthesis, or might represent a new wave of activity stimulated by the trauma of tissue cutting. The recovery of mitosis commences at about 5 hours, initially in the tissue edge, just when edge labelling is begining to decline. This, and the relative levels of I_s and I_m, suggests that separate cohorts of cells are being observed through two 'windows' in the cell cycle and that the peaks are too close together to represent a single cohort passing from S, through G_2 to the M phase, a process which should take at least 12 hours (Deschner & Lipkin, 1978).

The effects of growth control substances and trauma can be so rapid as to suggest a direct action on the control of cell movement from the G_2 to the M phase. During investigations on the physiology of mitosis in mouse ear epidermis *in vitro*, Gelfant (1959) noticed that mitotic activity was consistently influenced by trauma and not by culture techniques or nutritional requirements. He showed that a mitotic gradient appeared within five hours of cutting the epidermis *in vitro*; the response was not affected by plugging the cut with wax to exclude oxygen and nutrients. Since five hours is too short a period for cells to leave G_1 and pass through S and G_2 to the M phase, Gelfant (1962) and later Rajewsky (1965) proposed that some cells, already arrested in G_2, were stimulated into mitosis while other cells were switched into S from the G_1 phase within an hour. Other workers have noticed the rapid stimulation of DNA synthesis by trauma, for example in the regenerating liver by Bucher (1963) and in the cornea of rabbits by Harding & Srinivasan (1961), *in vivo* situations

where anoxia cannot be incriminated. The latter workers clearly showed that, following a small wound in the corneal epithelium, a wave of DNA synthesis, indicated by ^{3}HTdR labelling, commenced at 12–14 hours after the trauma and moved outwards in concentric rings at a rate of 17 μm per hour. Ten hours later, a wave of mitosis was apparent. Adding to the evidence that tritiated thymidine uptake here represented a predivision state was the fact that pairs of labelled nuclei, indicating daughter cells, appeared 24 hours after trauma. Wessels (1964) described similar phenomena in embryonic mouse pancreas in organ culture, but he explained these edge effects in terms neither of trauma nor central anoxia, but as a germ layer at the edge of the tissue fragments.

While traumatic stimulation of DNA synthesis and mitosis has most frequently been studied in the epidermis (Bullough, 1961; 1965), it has been observed in many other organs, and would seem to be a desirable homeostatic mechanism particularly in rapidly dividing tissue normally subjected to trauma. In the gastrointestinal tract, for example, McMinn & Johnson (1958) noticed large numbers of mitoses in the edges of wounds in the cat oesophagus after 48 hours while Block, Seiter & Oehlert, (1963) showed a labelling response in rat tongue within an hour of injury. Hunt (1958) showed similar changes in injured rat stomach.

It has been a common experience to see large rises in I_s shortly after explantation of tissue into culture, several times higher than the subsequent rise in mitotic index (Simnett & Heppleston, 1968), and the present experiments confirm this. Several mechanisms have been cited to explain this; for example, the larger duration of the *S* than the *M* phase, arrest in G_2 phase, or development of polyploidy and incorporation of ^{3}HTdR into metabolic DNA or even RNA (Maurer, 1981).

In the present study even when central labelling did return, the grain counts were much lower in central areas than in the periphery. The explanation for this may be the presence of a pool of endogenous 'cold' (unlabelled) thymidine, possibly from cell damage, in the centre of the tissue explants, causing a competitive inhibition of ^{3}HTdR uptake (Maurer, 1981).

The decline of labelling indices, and to a lesser extent mitosis, in the latter parts of the experiments may well represent limitations in the survival capabilities of colorectal mucosa in this particular system, or limitations of the labelling technique in organ culture, or both. The important observation is that kinetic changes are rapid and complex and that some central DNA synthesis does return at a time when the mitotic index is representative of *in vivo* values, and when the tissue appears to be histologically well preserved. Some of these changes may be due to lack of diffusion of essential nutrients, but trauma is also an important stimulus to cell division and this may be largely responsible for the edge effect in small explants. If colorectal mucosa in this particular organ system is allowed to settle for 12–24 hours, many of the difficulties that have thwarted previous attempts at *in vitro* kinetic studies disappear.

SUMMARY

In organ culture tissues undergo adaptive changes which complicate the assessment of proliferative indices. In the present study, a technique devised to preserve collagen metabolism and mesenchymal stroma in tissues of mixed origin was employed to investigate the kinetic behaviour of human colorectal mucosa over the first 24 hours in culture. During this period the structural preservation of tissue is good.

The mitotic index in mucosa is profoundly depressed throughout the explants during the first 2–6 hours in culture. During this same period, however, heavy uptake of tritiated thymidine, indicating DNA synthesis, is observed within 150 μm of the cut edge of the samples but not centrally. This phenomenon has been described previously and assumed to be the result of inadequate diffusion of oxygen into the tissue.

The 'edge labelling' phenomenon is followed by a wave of mitosis in the tissue edge at 8–18 hours in culture. This delay between tritiated thymidine uptake and the apppearance of mitoses in the tissue edge may simply represent the duration of the $S+G_2$ phase of the cell cycle, but is more likely to be due to cells in G_1 moving into the S phase followed by cells hitherto blocked in G_2 being stimulated into the M phase.

After 18 hours the mitotic and labelling indices become more uniform throughout the samples suggesting that 'edge labelling' is partly due to local stimulation of cell proliferation by trauma rather than central inhibition due to diffusion problems.

These results suggest that in this organ culture system, assessment of proliferative indices should be carried out after these adaptive changes have settled.

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Vindesine as a stathmokinetic agent in human rectal tumours in organ culture

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(*Received* 4 *July* 1984; *revision accepted* 3 *November* 1984)

Abstract. Organ culture, using human colorectal mucosa and tumours, is a good system in which to test a new stathmokinetic agent such as vindesine. Using this system we have found that vindesine has similar metaphase-arresting properties to vincristine, including at least a 6-fold dose response difference in its ability to arrest mitosis in mucosa and tumour, mucosa being the more sensitive. Vindesine is a satisfactory stathmokinetic agent, but in view of its greater cost offers no particular advantages over vincristine.

The stathmokinetic technique is one of a number of experimental methods used to study proliferation in cell populations. It takes advantage of the property of certain substances, notably the vinca alkaloids, to arrest mitosis in a form clearly visible by light microscopy, allowing cell birth rates to be calculated from metaphase accumulation with time. As an *in vivo* procedure in animals the technique has reached a high level of sophistication both in the normal gut (Wright, 1980) and in induced tumour models (Tutton & Barkla, 1976), but few studies have been carried out *in vivo* in human beings mainly because of ethical constraints (Camplejohn, Bone & Aherne, 1973; Wright *et al.*, 1977). Short term organ culture of human colorectal tissue has, however, proved a satisfactory system for studying metaphase arrest in more detail than previously possible (Pritchett *et al.*, 1982).

Vindesine (desacetyl vinblastine amide sulphate) is a semi-synthetic vinca alkaloid derived from vinblastine. Sweeney *et al.* (1978) noted that vindesine was a metaphase-arresting agent and observed some advantages over the related vinca alkaloids vincristine and vinblastine in growth inhibition in certain animal tumour test systems. These advantages included a wider spectrum of antitumour activity, a possible lack of cross resistance and less toxicity.

Vindesine was thus used clinically as a single agent against advanced human malignancy (Dyke & Nelson, 1977) in the hope that it would extend the therapeutic range of the vinca alkaloids, particularly in relation to gut tumours. Some useful activity was observed initially against colorectal carcinoma (Valdivieso, 1980), but later studies on advanced disease have proved disappointing (e.g. Saiers, Slavik & McKinney, 1982).

Hill & Whelan (1980) studied the pharmacokinetics of vindesine and vincristine. They showed similarities in that both were class II agents, exerting their lethal effects in the S phase in dividing cells and also in arresting mitosis. However, Sweeney *et al.* (1978) noted

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morphological differences in the appearances of nuclei arrested by vindesine and vincristine, suggesting possibly a different mode of action.

The properties of a substance suitable for stathmokinetic experiments have been defined by Tannock (1967); the essential principles of the technique were reviewed by Wright & Appleton (1980) and the advantages of an *in vitro* approach for the study of metaphase arrest in human tissue were discussed by Pritchett *et al.* (1982).

MATERIALS AND METHODS

Specimens from two patients undergoing surgical resection for rectal carcinoma were obtained directly from the operating theatre. Explants of normal-looking musoca and tumour roughly 2 mm × 1 mm were placed on micropore filters (Millipore Type SS 3·0 μM) and transferred in petri dishes with culture medium. The medium (Hodges & Melcher, 1976) consisted of Waymouth MB 752 medium (Gibco) containing 10–15% foetal calf serum and supplemented with 300 μg/ml hydrocortisone-21-sodium-succinate (Sigma), 0·45 μg/ml ferrous sulphate (B.D.H.), 100 units/ml penicillin/streptomycin solution (Gibco) and 100 units/ml mycostatin suspension (Gibco). The pH of the medium was checked and adjusted to 7·4 before filter-sterilization (Millipore Millex). The gas phase consisted of 95% O_2 and 5% CO_2. Tissue explants were carefully maintained at the fluid-gas interface and incubated at 38°C for 18 hr to allow the adaptive changes of explanation (Pritchett *et al.*, 1981) to abate.

Each resection specimen was used for a dose–response experiment. In the first experiment 10 Trowell jars were established, each containing roughly 20 explants, and 0, 0·1, 1·0, 5·0 and 10·0 μg/ml vindesine (Eldersine, Lilly) were added to 5 jars of tumour and 5 jars of mucosa respectively. Two explants of tumour and mucosa were taken every 30 min for 4 hr and fixed in neutral-buffered formal saline. After fixation the explants were processed to paraffin wax and serial sections were cut and stained with haematoxylin and eosin. Since a clear dose–response difference was apparent between mucosa and tumour, the second experiment was carried out to focus more accurately on the minimum dose of vindesine required for complete arrest in each tissue. For mucosa; 0, 0·2, 0·4, 0·6, 0·8 and 1·0 μg/ml vindesine were used, and for tumour; 0, 1·0, 2·0, 3·0, 4·0 and 5·0 μg/ml, sampling at 40 min intervals. Metaphase accumulation was counted in the same way in both experiments, carefully seeking evidence of normally-aligned and post-mitotic figures in the later parts of experiments. Mitoses were counted in a minimum of 2000 cells from two samples from each of two tissue explants giving four values for mitotic index per time point.

RESULTS

Accumulation of mitoses was apparent at all doses compared with the control and the morphological effect of vindesine on the mitotic apparatus was clearly visible. Arrested mitotic figures consisted of aggregations of grossly disordered chromosomes, roughly spherical in shape; on degeneration, these figures became dark, globular and homogeneous. The appearances, by light microscopy, were indistinguishable from those caused by vincristine. Throughout the experiments, both tumour and mucosa were morphologically well preserved.

Mitotic index (I_m) was plotted isometrically against time and dose of vindesine in both experiments (Fig. 1. a–d). In the first dose–response experiment, the lowest dose at which no metaphase escape was apparent was 1·0 μg/ml for mucosa and 5·0 μg/ml for tumour. In the second experiment, the area below these 'minimum' doses was explored. In mucosa, the minimum dose of vindesine for complete arrest was 0·8 μg/ml whereas in tumour, even at 5·0

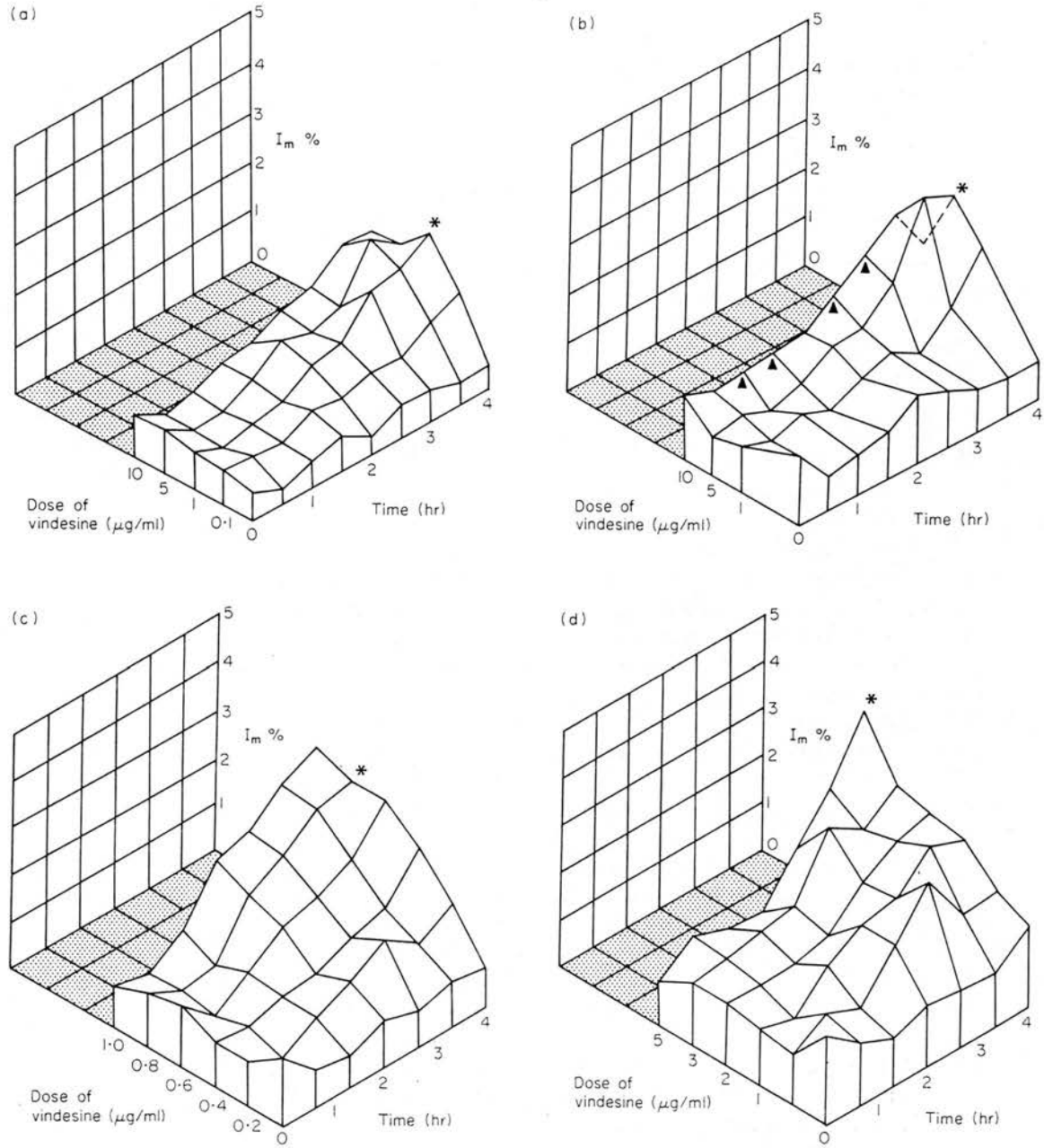

Fig. 1. Three-dimensional isometric plots of two dose–response experiments. (a) Experiment 1. mucosa, (b) Experiment 1. tumour, (c) Experiment 2. mucosa, (d) Experiment 2. tumour. Each point represents mean value of I_m from four counts of >2000 cells from two tissue explants.

* = minimum dose for complete metaphase arrest.

▬ = missing data.

x = time (hr)

y = (μgm/ml vindesine)

z = I_m% (mitotic index)

μg/ml, although no actual anaphases were seen, many suspiciously well-aligned mitotic figures were apparent. Mitotic index was increased between four and five times after 4 hr with vindesine, at the minimum doses for complete arrest.

Data for the 0·1 μg/ml dose of vindesine in tumour is absent from Fig. 1 because tumour tissue was not present in the explants.

Estimates of birth rate calculated by regression analysis of the mitotic index data for the two optimum doses of vindesine, assuming a rectangular age distribution, were: 10·0 $\pm$ 2·7 cells/1000 cells/hr for tumour and 9·4 $\pm$ 1·0 cells/1000 cells/hr for normal mucosa.

DISCUSSION

It has been confirmed that vindesine can arrest mitoses in a manner that is morphologically apparent in standard histological preparations. Arrested mitoses can easily be counted over a wide range of doses but there is an optimum dose for complete arrest. Mitotic degeneration does not occur before 200 min with vindesine and no apparent toxicity occurs to non-dividing cells within this short experimental period. These features comply with the criteria of Tannock (1967) for evaluating stathmokinetic agents.

The action of vindesine appears very similar to that of vincristine and at ×400 magnification the mitoses arrested by the two drugs are indistinguishable. Sweeney *et al.* (1978), comparing the action of vinblastine and vindesine on Chinese hamster ovary cells in culture, described 'dispersed chromosomes' with vindesine, but at the same time, they suggested that vindesine-binding to tubulin was similar to vincristine and vinblastine although there were possibly some quantitative differences in binding affinity.

The advantages of using organ culture for carrying out stathmokinetic experiments on human tissue are considerable. Dose–response experiments, requiring many tissue samples, are essential for identifying minimum doses of drug required for complete metaphase arrest, since higher than optimal doses may well arrest or even kill cells in S or even G_2 so tending to reduce estimates of birth rate by impeding entry into mitosis (Fitzgerald & Brehaut, 1970). In addition the presence and duration of the 'lag' and 'plateau' phases can be investigated by multiple tissue sampling at short time intervals, impossible to achieve *in vivo*.

Using such an approach, a clear dose–response difference is apparent, as with vincristine (Pritchett *et al.*, 1982), and at least a 6-fold difference exists between tumour and mucosa in the dose of vindesine required for complete metaphase arrest. The exact reason for this difference is not known but there is accumulating evidence from animal tumour test systems that vincristine is actively extruded from tumour cells in a way that can be reversed by inhibitors of the slow channel of Ca^{2+} transport (Tsuruo *et al.*, 1983). Vindesine shows similar 'lag' and 'plateau' phases to vincristine and the linear section of mitotic accumulation (from which cell birth rate can be calculated) is easily identified. In both mucosa and tumour in the first dose–response experiment, there is a suggestion that high doses of vindesine cause not a 'plateau' in values for I_m but a decline at 4 hr possibly due to toxicity.

Birth rates for mucosa and tumour are not significantly different using vindesine, as seen with vincristine (Pritchett *et al.*, 1982), but were calculated from a single experiment.

In conclusion, vindesine is a satisfactory stathmokinetic agent but in view of its higher cost, offers no particular advantage over vincristine.

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Short Communication

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Verapamil increases the sensitivity of primary human colorectal carcinoma tissue to vincristine

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The inherent resistance of human solid tumours to cancer chemotherapy is a major problem in medical oncology. Experimentally, in cell lines, the resistance phenomena studied are often induced by a process analogous to the induction of antibiotic resistance in bacteria. The acquired mechanisms by which resistant mutants differ from parent cells are studied and less attention paid to the differences between the innate resistance of a tumour and the normal cell population from which it arose. There is an urgent need to relate the results obtained in these systems to human tumour tissue.

Verapamil, a calcium transport antagonist widely used in cardiological medicine, has been shown to be a potent modifier of vincristine resistance in several cell lines (Tsuruo *et al.*, 1981 & 1983). Clinical trials using verapamil as a modifier of vindesine therapy in advanced human malignancy are in progress (Cantwell *et al.*, 1985). In the present study we have examined the effects of verapamil on the sensitivity of primary human colonic carcinomata to vincristine.

In the formal stathmokinetic experiment a dose of vincristine is administered, large relative to clinical therapy (i.e. approximately 15 times greater for human colon cancer), to arrest all dividing cells in metaphase. The resulting "arrested metaphase" figures are easily recognised in histological preparations, and their rate of accumulation is used to calculate the cell birth rate. Using this technique we have previously demonstrated that a six times greater dose of vincristine is required to achieve a maximum rate of metaphase accumulation in human colonic tumour tissue than that required for normal colonic mucosa (Pritchett *et al.*, 1982). We have recently developed a method of measuring directly the degree of escape from metaphase arrest by counting the proportion of mitotic figures showing anaphase or telophase configuration (Ince *et al.*, 1985). The resulting Post-Metaphase Index (PMI) can be used in a "dose-response" format at a range of vincristine doses extending down to the human therapeutic range (i.e. 200–300 nmol plasma levels). This is the technique we have used in assessing changes in vincristine resistance in the present study.

Eleven human colorectal carcinomata were studied. All were left-sided lesions treated by abdominoperineal resection, anterior resection, or left hemicolectomy. The specimens were collected and cleaned in theatre, and transported to the laboratory in ice-cold medium. Apparently viable areas of tumour tissue were identified, from which explants, measuring $\sim 2\,mm^3$, were prepared. The explants were placed on millipore filters in 60 mm plastic petri dishes and were cultured in Weymouth's MB752/1 medium supplemented with 10% foetal calf serum, hydrocortisone, vitamin C, and ferrous sulphate. The dishes were maintained in a controlled atmosphere chamber with a gas phase of 95% O_2, 5% CO_2, using a rocking culture technique (Senior *et al.*, 1984).

Altogether 120 explants per tumour were used with four explants to each dish. After 16 h incubation the dishes were divided into three groups and the medium was changed to include verapamil at one of three doses *viz.* 0.0 μmol, 6.6 μmol, 13.2 μmol. After a further 2 h the media were again changed to include verapamil at the same dose and vincristine at one of five doses, *viz.* 270 nmol, 540 nmol, 1080 nmol, 2160 nmol, 4320 nmol. After 2 h incubation with vincristine the tissues were fixed in formalin and routinely processed to paraffin wax. Histological sections were prepared at 4 μm and stained with haematoxylin and eosin.

The PMI was derived by evaluating a total of at least 50 tumour cell mitotic figures in step sections of each explant. Multiples of a whole section of each explant were counted including that in which the 50th mitosis was identified. The proportion of post-metaphase mitotic figures was recorded. At each combination of verapamil and vincristine doses the data from the several explants were pooled to provide a mean PMI. Out of eleven tumours six were rejected prior to counting because

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Received 17 July 1985; and in revised form 12 September 1985.

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of unsuccessful culture; failure was due to bacterial contamination, tumour necrosis, or the fact that some tumours were scirrhous with much stroma and little epithelial tissue. From the five successful tumour cultures 45% of explants were unsuitable for counting, owing to excessive necrosis, or the presence of only minimal epithelial tumour tissue. Some of the "unsuitable" tumour explants comprised normal colonic mucosa only.

The five tumours which were cultured successfully were all graded histologically as moderately differentiated adenocarcinomata. Two were from the sigmoid colon and three from the rectum. Dukes' staging was as follows: stage A – 1 tumour; stage B – 2 tumours; stage C – 1 tumour; stage D – 1 tumour.

The pooled data from all five tumours are shown in Table I. Figure 1 is a three-dimensional plot of the relationship between the PMI (%) and vincristine dose at the three verapamil doses used. At all three doses there is an increase in metaphase escape with decreasing dose of vincristine, and this corresponds with our previous observations on this type of data. Verapamil at doses of both 6.6 μmol, and 13.2 μmol, comparable with therapeutic plasma levels up to 10 μmol (Cantwell *et al.*, 1985) causes a similar and statistically significant degree of enhancement of the effect of vincristine. No simple function of the PMI is linearly related to dose or log dose of vincristine. Analysis using the GLIM programme (Baker & Nelder, 1978) and a logistic transformation of the PMI shows that either dose of verapamil reduces the PMI by a factor of 0.61 (95% confidence limits 0.47 to 0.78, $P<0.001$) independently of the dose of vincristine. A satisfactory fit ($\chi^2_9=10.91$) is found by fitting the model;

Logit PMI = $-6.34-0.50$ if verapamil
(s.e. 0.13 $P<0.001$)
$+0.00$ if 4320 nmol of vincristine
1.01 if 2160 nmol of vincristine
1.27 if 1080 nmol of vincristine
3.05 if 540 nmol of vincristine
3.35 if 270 nmol of vincristine

These results provide direct experimental evidence of the efficacy of pharmacological modification of primary solid human tumour resistance to vincristine.

Acquired tumour cell resistance to vincristine is frequently associated with cross-resistance to anti-cancer drugs of differing modes of action, notably adriamycin. This has been termed the pleiotropic multidrug-resistance phenotype, and in animal tumour-cell lines appears to be related to the presence of, and phosphorylation status of, a cell

Table I Mean Post-Metaphase Index with standard errors.

vincristine dose (*nmol*)	*verapamil dose* (μ*mol*)		
	0.0	*6.6*	*13.2*
270	4.6±1.3	2.9±0.7	3.3±1.5
540	3.4±1.4	2.6±0.6	2.0±0.6
1080	2.4±1.4	0.5±0.3	1.0±0.6
2160	0.5±0.2	0.3±0.3	0.2±0.1
4320	0.0	0.1±0.1	0.3±0.3

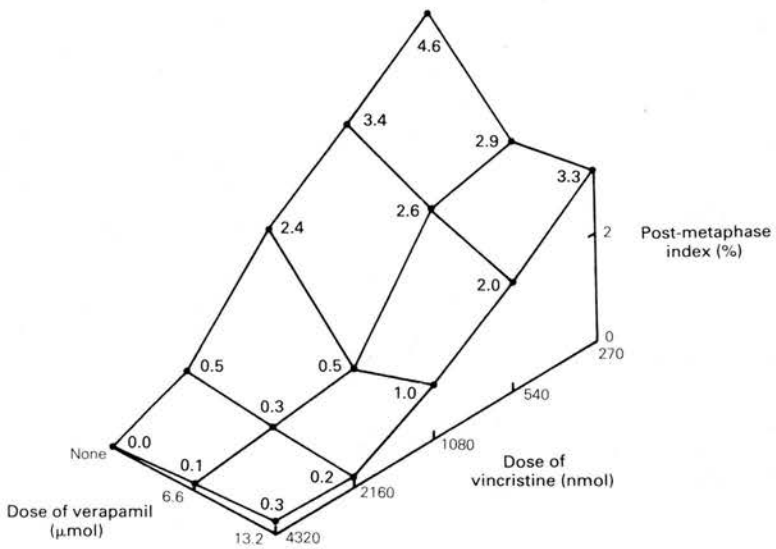

Figure 1 Three dimensional plot of the effects of vincristine and verapamil on the postmetaphase index.

surface glycoprotein designated P180 (Ling *et al.*, 1983; Garman *et al.*, 1983). A wide range of pharmacological modifiers of both vincristine and adriamycin resistance have been described in a variety of cell lines of human and animal origin displaying either acquired or inherent drug resistance. The pharmacologically induced increase in tumour-cell sensitivity is accompanied, and possibly caused, by increased intracytoplasmic accumulation of the anticancer drug (Tsuruo *et al.*, 1982). Thus the postulated mechanism of resistance is the existence of a drug elimination pathway in the plasma membrane which allows cancer cells to minimise the intracellular concentration of the drug. Verapamil is thought to inhibit this pathway. However, other possible resistance mechanisms, such as increased intracytoplasmic drug binding may operate (Beck *et al.*, 1983), and the issue remains to be resolved. Currently we are using our organ culture system to investigate the underlying biochemical basis of vincristine resistance in primary colonic cancer. This is of crucial importance in the longer term development of more specific and potent modifiers for use in clinical therapy. Current experimental strategies comprise empirical selection of potential modifiers of vincristine resistance, and battery testing against a range of cell lines of varying drug resistance profile. This approach has demonstrated that different agents vary in their ability to act as modifiers depending upon the particular combination of cytotoxic drug and cell line tested (Ramu *et al.*, 1984). It is clear from our results that there is evidence *in vitro* that verapamil has a modifying effect on human primary colonic cancer cells. This may be of some clinical usefulness in the future, but verapamil will not necessarily prove to be the most effective enhancer of vincristine potency.

The verapamil used was kindly donated by Abbott Laboratories, Queenborough, Kent. We are grateful to Kathryn Elliott for the preparation of histological sections and to Brenda Kennedy who typed the manuscript. This study was supported by a grant from the North of England Cancer Research Campaign.

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Cell Tissue Kinet. (1973) **6**, 239–246.

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CELL PROLIFERATION IN THE CASTRATE MOUSE SEMINAL VESICLE IN RESPONSE TO TESTOSTERONE PROPIONATE

I. EXPERIMENTAL OBSERVATIONS

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(*Received* 8 *August* 1972; *revision received* 4 *November* 1972)

ABSTRACT

The cell proliferation kinetics following induced DNA synthesis in the mouse seminal vesicle were measured after treatment with testosterone propionate. Fraction labelled mitosis curves at 24, 48 and 72 hr after injection gave t_2 values of 1·5, 2·0 and 1·8 hr respectively, and t_s values of 10·5, 8·0 and 8·0 hr. T_c measured 48 hr after stimulation was 17·5 hr. Growth fraction rose from 0·14 at 24 hr to 0·64 at 48 hr, and fell to 0·32 by 72 hr. A simple model is proposed in which the rise and fall of mitotic index and labelled index is determined by the 'cell distribution ratio'.

INTRODUCTION*

The prostatic complex of the male rodent is composed of seminal vesicles, coagulating glands, dorsal, ventral and lateral prostates. The cell proliferative response in the prostatic complex of the castrate male which follows androgen treatment has been widely studied (Burkhardt, 1940; Allen, 1956, 1958; Coffey, Shimazaki & Williams-Ashman, 1968; Ritter, 1969). The main features of the response are a latent period of 15–20 hr, then a rapid increase in DNA synthesis followed by a wave of mitoses. If the mitotic index (I_m) and the labelling index (I_L) are observed under continued hormonal treatment, they show a gradual decline over 100–150 hr. At the same time there is evidence of functional differentiation in cellular hypertrophy, cytoplasmic vacuolation and the production of secretion. The response of the prostate to androgen is an example of stimulated DNA synthesis comparable to the oestrogen stimulated endometrium (Epifanova & Terskikh, 1969), the liver after partial hepatectomy (Grisham, 1962) or the carcinogen stimulated bladder epithelium (Levi, Cowan & Cooper, 1969).

* Abbreviations: T_c, duration of the cell cycle; t_s, duration of DNA synthesis; t_{G1}, duration of the pre-synthetic period; t_{G2}, duration of the post-synthetic period; t_m, duration of mitosis. $t_2 = t_{G2} + \frac{1}{2}t_m$. I_p = growth fraction.

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The events which precede mitosis in models of stimulated synthesis have been extensively investigated. The finding that differing cells upon stimulation pass through similar biochemical events suggests that they originally exist in a common metabolic state which has been termed G_0. It has been suggested that it is characteristic of G_0 cells that mitosis precedes differentiation (Epifanova, 1966). Alternatively these quiescent cells may be in a long premitotic (G_1) compartment (Lajtha, 1963). Both differentiated cells (hepatocytes) and non-functional cells (i.e. prostatic or endometrial cells in castrates) show a similar latent period, suggesting that once the decision to undergo cell division has been made the state of differentiation is not a limiting factor in the onset of DNA synthesis. Despite similar proliferative potential in G_0 cells specific biochemical findings are scanty. However, Schwartz *et al.* (1965) and Farber & Baserga (1969) have shown that *in vivo* hydroxyurea caused necrosis after inhibition of DNA synthesis in the small intestine whereas there was only a transient inhibition of stimulated DNA synthesis in the liver, kidney and salivary gland.

In the castrate mouse seminal vesicle Tuohimaa & Niemi (1968) have shown that the duration of DNA synthesis is considerably shortened after treatment with testosterone. However, preliminary studies (Morley & Wright, 1972) indicate that the gradual fall in I_m and I_L following the hormone induced wave of proliferation may be explained by alteration of the proportion of cells in the proliferative cycle rather than by changes in the duration of cell cycle parameters.

It is the purpose of this communication to determine the duration of cell cycle parameters at various times during the course of a stimulated proliferative response, and to propose a simple model of the response in terms of these parameters.

MATERIALS AND METHODS

Male Balb/c mice aged 3 months were castrated. Two weeks later, between 09.00 and 10.00 hours, each animal was injected subcutaneously with 250 μg of testosterone propionate (Koch-Light, Colnbrook, Bucks., England) dissolved in sesame oil. Thereafter testosterone propionate was injected every 24 hr until the animal was killed.

To obtain values for I_m and I_L throughout the period of the stimulated cell proliferation animals were killed by cervical dislocation at intervals of 3 hr for up to 100 hr after the initial injection of testosterone propionate. Tritiated thymidine (specific activity 5·0 Ci/mm; Radiochemical Centre, Amersham) was injected intraperitoneally at a dosage of 1 μCi/g 1 hr prior to death. At autopsy the seminal vesicles were fixed in Carnoy's solution. Multiple sections were prepared at a thickness of 5 μm. Autoradiographs were prepared using Kodak A.R. 10 stripping film and developed in Kodak D19b. Sections were exposed for 2 weeks and stained with Harris's haematoxylin. All cells with more than 5 grains per nucleus were counted as labelled.

To obtain estimates of I_m and I_L a total of 2000 cells were counted at the distal ends of the seminal vesicles from each animal. The proportions of mitotic and labelled cells were expressed as percentages.

The cell cycle parameters were estimated by the fraction labelled mitoses method (FLM) starting at 24, 48 and 72 hr after the initial injection of testosterone propionate. At these times tritiated thymidine was administered by the route and dosage given above. Thereafter animals were killed at approximately hourly intervals for up to 35 hr. In obtaining the FLM curves 100 mitotic cells in all stages of division were counted, except in a few indicated samples

where I_m was very low. The median durations of cell cycle parameters were measured from the FLM curves at the 50% level (Quastler & Sherman, 1959).

RESULTS

The variation of I_L with time after the first injection of testosterone propionate is shown in Fig. 1. After a quiescent period of 20 hr there is a rapid increase in I_L to values greater than 25% at 35–45 hr. I_L then diminishes to 5% at 90 hr. There is no apparent relationship between I_L and the injection of testosterone propionate at 24, 48 and 72 hr.

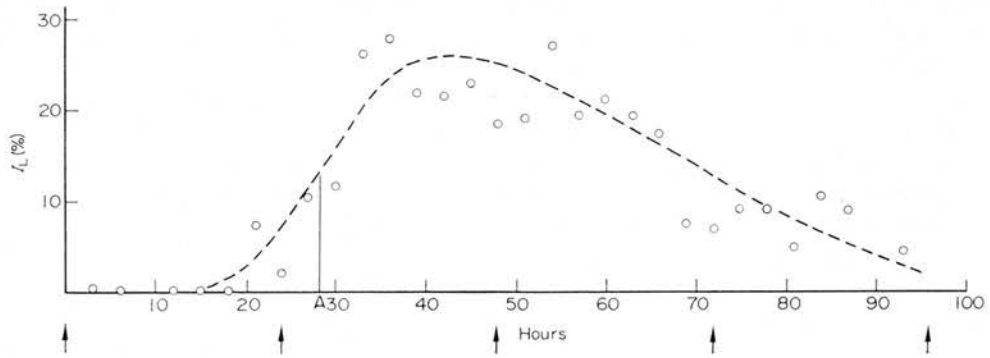

FIG. 1. I_L in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg testosterone propionate. Arrows show times of subcutaneous injections. The 50% peak value is indicated by the line A.

The variation of I_m with time in the same animals is shown in Fig. 2. The quiescent period is approximately 30 hr, followed by a rise to a maximum of 1·8% at 48 hr. I_m then gradually falls to 0·4% at 90 hr. Additional values for I_m may be added from animals used in FLM estimates of cell cycle parameters. The longer period of exposure to tritiated thymidine does

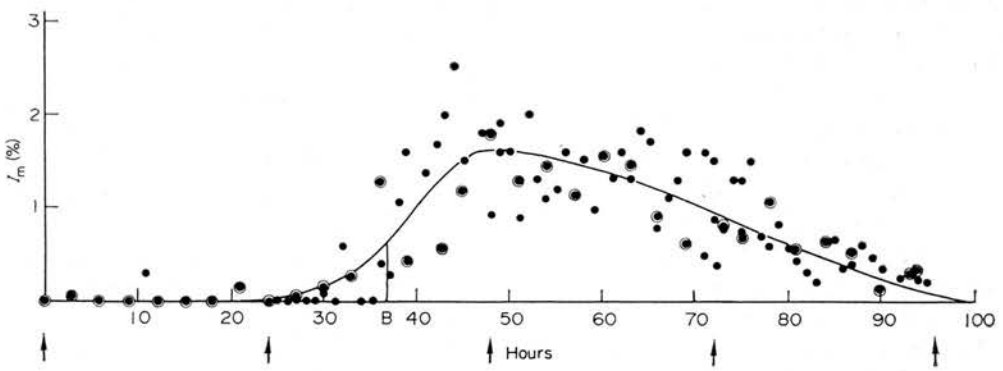

FIG. 2. Encircled points: I_m in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg testosterone propionate. Arrows indicate times of subcutaneous injections. •, Additional I_m values from animals used in FLM estimations. The line B indicates the 50% peak value.

not appear to influence I_m, or alter the general form of the I_m response. This composite curve is the basis of all further discussion of I_m. The points tend to form a plateau between 50 and 70 hr. The scatter of I_m increases with the level of I_m since such indices are binomially distributed. The rising portions of the I_L and I_m curves indicate the passage of a cohort of cells into DNA synthesis, and into mitosis. Taking the difference in time between the 50% peak values (A, B) of the two curves gives an estimate of $t_s + t_{G2}$.

$$t_s + t_{G2} = 9{\cdot}5 \text{ hr}$$

FLM curves

Fig. 3 shows the FLM curves obtained after the injection of tritiated thymidine at 24, 48 and 72 hr.

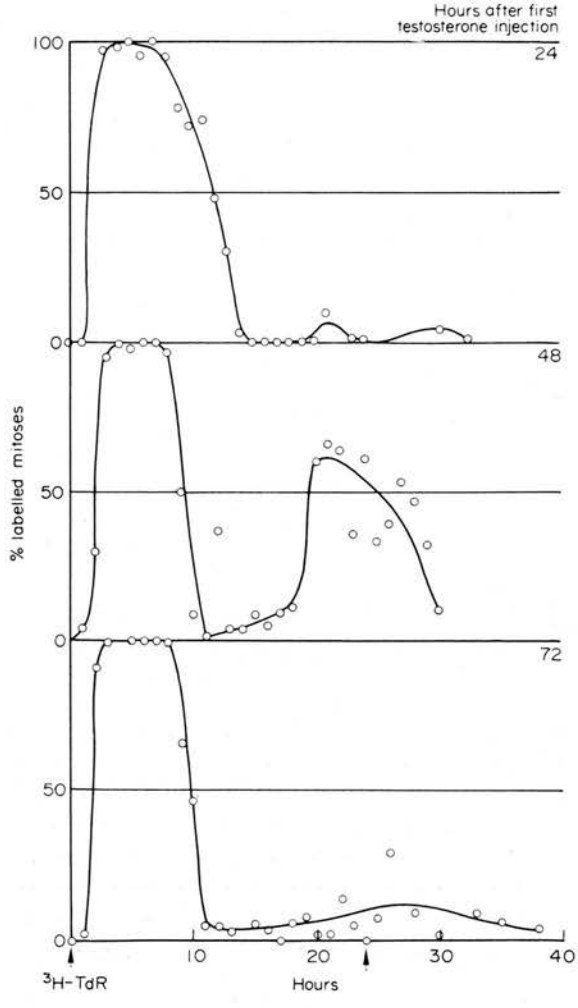

FIG. 3. FLM curves in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). Tritiated thymidine given 24, 48 and 72 hr after initial androgen stimulation.

24 hr after testosterone propionate injection I_m is low, although I_L is increasing. For this reason it was not possible to count more than ten mitoses in each sample during the first 5 hr of the FLM curve. The value of t_2 is 1·6 hr, and of t_s is 10·5 hr. The FLM falls rapidly to zero some 15 hr after injection of ^{3}H-TdR, and there is no evidence of a second wave of labelled mitoses up to 33 hr. This might indicate that the labelled cohort divides only once. However, examination of Figs. 1 and 2 show that a much greater proportion of the population enters DNA synthesis after the labelled cohort. It is probable that the second division of labelled cells is masked by unlabelled cells.

From the FLM curve obtained after 48 hr testosterone propionate treatment, t_2 is 2 hr, t_s is 8 hr, and T_c is approximately 17·5 hr. The rapid rise and fall of the first wave of the FLM curve demonstrates that there is relatively little variation in t_s. The presence of a distinct second wave indicates that many of the labelled cohort have undergone a second division. Throughout this period I_L and I_m have fallen slightly, but the rate of change (see Figs. 1 and 2) is much less than in the FLM curve performed 24 hr after stimulation. Hence distortion of the FLM curve by unlabelled cells is probably less.

The FLM curve at 72 hr after stimulation gives a t_2 of 1·8 hr, t_s of 8 hr, but no direct estimate of T_c. The well-defined first peak indicates little variation in t_s. No evidence of a second peak was found up to 38 hr after tritiated thymidine injection. The low values of I_L (Fig. 1) during the course of this FLM curve indicate that the proportion of cells in the proliferative cycle has diminished, since there has been no large alteration in t_s.

TABLE 1. Duration in hours of cell cycle parameters in the seminal vesicle of castrate male Balb/c mice after androgen stimulation

Hours after androgen stimulus	Cell cycle parameter				
	t_2	t_{G1}	t_s	T_c	I_L (%)
24	1·6	—	10·5	101*	7·5
48	2·0	7·5	8·0	17·5, 25·7*	25
72	1·8	—	8·0	48·2*	12·5

* Calculated from I_L and t_s assuming exponential growth following the equation of Cleaver (1967):

$$I_L = (\exp t_s \ln 2/T_c - 1)\exp t_2 \ln 2/T_c \qquad (1)$$

The cell cycle parameters estimated from the FLM curves are summarized in Table 1. Accurate values for t_m cannot be estimated from FLM curves. The rapid rise of the first peak of the FLM curves suggest t_m values of about 1 hr. Calculated values of T_c are large, and even 48 hr after stimulation, the calculated T_c is considerably longer than the observed T_c. The data in Table 1 indicates that if these calculated values of T_c are accepted then a labile t_{G1} must be postulated. An alternative hypothesis is that at the beginning and end of the proliferative response a proportion of the population is not in cycle, i.e. the growth fraction (I_p) is less than 1. A value for I_p may be calculated from the relationship:

$$I_p = \frac{I_{L\ \text{observed}}}{I_{L\ \text{theoretical}}} \qquad (2)$$

in which $I_{L\ \text{theoretical}}$ is calculated from equation (1). If it is assumed that the T_c of 17·5 hr

observed 48 hr after androgen stimulation is applicable at 24 and 72 hr, the observed changes in I_m and I_L may be explained by alteration in I_p (see Table 2).

TABLE 2. Growth fraction (I_p) in the seminal vesicle of castrate male Balb/c mice after androgen stimulation

Hours after androgen stimulation	$I_{L\,observed}$ (%)	$I_{L\,theoretical}$ (%)	I_p
24	7·5	53·7*	0·14
48	25	39·4	0·64
72	12·5	38·6*	0·32

* T_c assumed to be 17·5 hr.

DISCUSSION

The proliferation following androgen stimulation of the seminal vesicles of the castrate male Balb/c mouse is similar to that in other models of stimulated DNA synthesis (Cooper, 1971). It has been suggested that prior to stimulation the cells in these models are in a common state of preparedness for entry into the proliferative cycle. In target tissues such as the endometrium of the castrate female or the accessory sex glands in the castrate male sex hormones produce a marked proliferative response. The cells of the mouse seminal vesicle of a castrate may be regarded as belonging to a G_0 population.

The cell cycle parameters of this G_0 population are becoming apparent. It is generally agreed that I_m and I_L are even lower than in intact control animals (Morehead & Morgan, 1967; Allen, 1958). It has been shown that t_s in the ventral prostates of castrate mice is shortened by androgen treatment (Tuohimaa & Niemi, 1968). Cell cycle measurements are not feasible in tissues of very low I_m. The total number of cells in the accessory sex glands is probably considerably diminished after castration; Kochakian (1963) demonstrated that 14 days after castration the total DNA of the mouse ventral prostate fell nearly 40%. Even greater changes in total gland weight and RNA were found, reflecting the loss of functional activity. No direct measurements on cell number following castration have been performed.

Kochakian & Harrison (1962) restored ventral prostate DNA to pre-castration levels within 5 days by the implantation of testosterone propionate. DNA levels remained constant for up to 40 days thereafter despite continued androgen treatment. The fall in I_m and I_L values observed in the present experiments is consistent with cessation of DNA synthesis. If growth fraction considerations are not taken into account, the cell cycle parameters estimated for FLM curves (i.e. t_s, t_2 and T_c at 48 hr) cannot be reconciled with observed values of I_m and I_L except by postulating an extremely labile t_{G1}.

If T_c is assumed to remain substantially constant, the rise in I_m and I_L may be explained by the entry of cells into the proliferative cycle from a G_0 compartment. After each cell cycle a proportion of daughter cells leave the proliferative cycle and I_p diminishes. The finding of a fall in I_L in the absence of large changes in t_s also indicates a fall in I_p. Tannock (1968) observed a similar phenomenon in a transplanted mouse mammary tumour which maintained a constant T_c in anoxic areas; although the growth fraction and hence I_m diminished. Although calculations of I_p based on exponential equations are clearly not ideal during a transitory proliferative response, the results (Table 2) are consistent with this hypothesis.

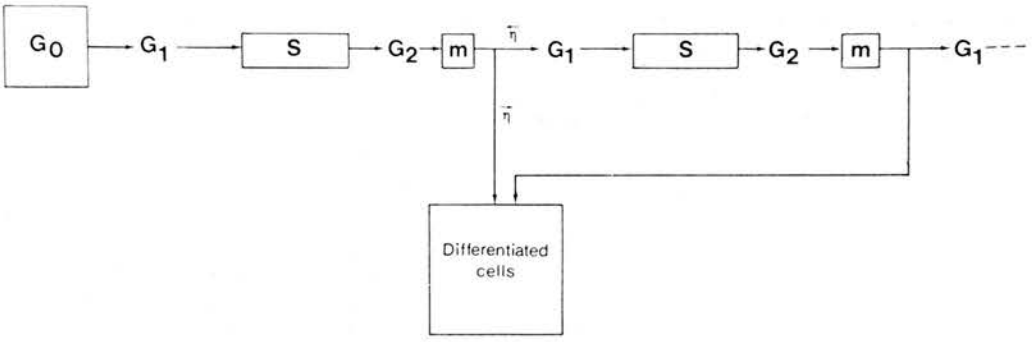

FIG. 4. Simple model of stimulated cell kinetic response in seminal vesicle of castrated male Balb/c mice following daily testosterone propionate treatment. $\overleftarrow{\eta}$ is the probability of remaining in the proliferative cycle, $\overrightarrow{\eta}$ is the probability of leaving the proliferative cycle.

Fig. 4 illustrates the postulated flow of cells from G_0 through the cell cycle. The wave of cells passing into DNA synthesis and mitosis (Figs. 1 and 2) suggests considerable variation in the rate of efflux of cells from G_0, or variation in the duration of the first t_{G1}, or a combination of these two factors. The square form of the FLM curves (Fig. 3) indicates little variation in t_s, t_2 and t_m. It therefore seems probable that rate of efflux from G_0, and variations in the first t_{G1} are the major sources of variation within the system.

The most probable site for the decision on whether to remain in the proliferative cycle or to enter the differentiated compartment is after mitosis. Previous evidence (Morley & Wright, 1972) using grain count distributions showed that all cells labelled at the beginning of an FLM experiment divided, but that only a small proportion of daughter cells went into mitosis. Bresciani (1968) introduced the concept of a 'distribution parameter' or cell distribution ratio (d-ratio) which he defined as the ratio of the proportion of new cells remaining in the proliferative cycle ($\overleftarrow{\eta}$), to the proportion of cells differentiating or leaving the cycle ($\overrightarrow{\eta}$). The probability of remaining in the cell cycle at any time is $\overleftarrow{\eta}$ and the probability of leaving the proliferative cycle is $\overrightarrow{\eta}$. The values of these probabilities have a profound influence on the growth fraction. The present data suggests that the form of I_m and I_L curves during the induced cell proliferation may be determined by the value of the probability of remaining in the cell cycle.

ACKNOWLEDGMENTS

We are grateful to Professor A. G. Heppleston for continued encouragement and advice. Miss Doreen Hardy and Mr H. Elliot rendered skilled technical assistance. We gratefully acknowledge the financial support of the North of England Branch of the Cancer Campaign.

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CELL PROLIFERATION IN THE CASTRATE MOUSE SEMINAL VESICLE IN RESPONSE TO TESTOSTERONE PROPIONATE

II. THEORETICAL CONSIDERATIONS

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(*Received* 8 *August* 1972; *revision received* 26 *October* 1972)

ABSTRACT

The cell cycle kinetics of a conditional renewal system which has been stimulated by a continuing supply of hormone were investigated by means of a model, basic to which is the concept of decycling probability. The integral equations of the system were formulated and solved numerically in an environment of interaction with the computer. Graphical output on a visual display screen was used to facilitate the extraction of information from the model, and its consistency with experimental data was demonstrated.

INTRODUCTION

If a conditional renewal system is deprived of a necessary hormone, mitotic activity is reduced to very low levels and the cells may be considered to be in a G_0 or long G_1 phase. After hormonal stimulation there is a delay followed by a burst of DNA synthesis and mitosis which then dies out (Morley & Wright, 1972). This is so even if the hormone is ensured to be in plentiful supply by administering it at regular intervals (Morley, Wright & Appleton, 1973). Where experimental data is referred to it is taken from this latter article. The following model is proposed to describe the system (Fig. 1).

Cells start in compartment G_0 and after stimulation some or all of them pass through G_1, S, G_2 and M phases. After mitosis a daughter cell may either leave the cycle and enter the differentiated compartment or undertake a further cell cycle. The number of cells taking these alternative paths is controlled by the 'decycling probability'. The idea of decycling probability is akin to Bresciani's distribution ratio (Bresciani, 1968) and corresponds to his $\bar{\eta}$. It is more basic and for many purposes more useful than the growth fraction, particularly when phase distribution is uncertain, although both quantities give the same kind of information about the system.

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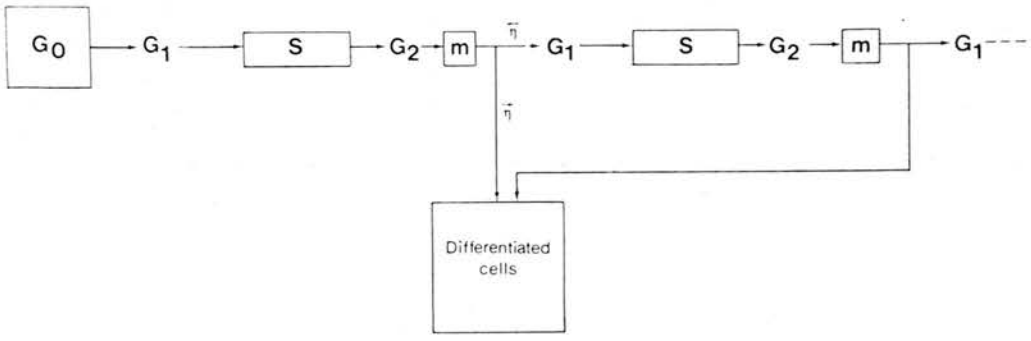

FIG. 1. Simple model of stimulated cell kinetic response in seminal vesicle of castrate Balb/c mouse following daily testosterone propionate treatment.

Simulations of the cell cycle have gained acceptance in recent years since Barrett's Monte-Carlo approach (Barrett, 1966) and Takahashi's investigation of a stochastic equation technique for analysis of FLM curves (Takahashi, 1966) were reported. The model described in Fig. 1 was treated analytically because of the greater speed attainable and because more insight can be obtained from a mathematically defined model than from a Monte-Carlo model, which often tends to mask the important effects by modelling too closely the noise (random or other irrelevant variation) of the system under investigation. Graphical presentation of output was chosen because of its inherent advantage in ease of understanding and communication.

USE OF THE MODEL

Unlike most FLM curve-fitting techniques, designed to estimate the durations of the phases of the cell cycle, the present model is regarded primarily as a means of gaining familiarity with and insight into the system it represents. It is true that it can be used to estimate the cell-cycle parameters, and in fact a mitotic index curve provides a better estimate of the duration of mitosis than can be obtained from a FLM curve. But a mathematical model should do more than estimate parameters; it should also teach its users about the system it represents. Furthermore, a good model will be amenable to verification by suggesting other experiments. Decycling probabilities found from the model can be examined by means of grain-count experiments, and the total number of cells in the system at any time can be compared with DNA measurements.

In an examination of the effect on the final population size of changing the decycling probability or of postulating different quantities upon which it might depend, the user of the model will require to carry out several simulation runs. Frequently he will choose the parameters for one run only after seeing the results of the previous run. If such a procedure is to be carried out conveniently it is important that the computing facilities available should support man–machine interaction at a fairly sophisticated level. The model under discussion was programmed in ALGOLW with FORTRAN graph-plotting routines, and was designed to be run from an IBM 2741 typewriter terminal on an IBM 360/67 supporting MTS (Michigan Terminal System). Graphical output was produced on a Computek 400 storage

display screen connected to the IBM 360 via a PDP-11. This latter machine also supported the Benson-Lehner graph plotter which provided hard copy output when a particularly informative set of parameters was used, or when a fit was obtained for experimental data.

With such equipment the time elapsing from the start of entering data in response to prompting from the program to the completion of the display of labelled and mitotic index curves was about 3 min, the exact time depending on the pressure on the time-sharing system from other users. The time for such a run was always less than that taken up by discussion of its output.

FITTING THE CURVES TO DATA

All indices which can be expressed as the proportion of 'successes' in a number of 'trials' may be treated identically in the matter of fitting curves to observed values. Thus the following argument applies equally to labelling index, mitotic index, cumulative labelling index and FLM curves.

Suppose the experimental index at time t is λ_t, derived from v_t observations, and the value predicted by the model is μ_t. For the FLM curve several methods are in use. Takahashi, Hogg & Mendelsohn (1971) minimize the weighted sum of squares

$$\sum_t v_t(\lambda_t - \mu_t)^2$$

which does not take into account that for the same number of observations the sampling variance is higher near $\lambda_t = 0{\cdot}5$ than it is near 0 or 1. MacDonald (1970) maximizes the log-likelihood

$$\sum_t v_t\{\lambda_t \log \mu_t + (1 - \lambda_t)\log(1 - \lambda_t)\}$$

which suffers because μ_t may be zero, and he has suggested to Steel & Hanes (1971) that they minimize

$$\sum_t (\arcsin\sqrt{\lambda_t} - \arcsin\sqrt{\mu_t})^2$$

This last technique takes the differences in sampling variance into account, but we prefer to extend it slightly so that the possibility of counting different numbers of cells is catered for, and consider the expression

$$\sum_t v_t(\arcsin\sqrt{\lambda_t} - \arcsin\sqrt{\mu_t})^2 \qquad (1)$$

The authors cited use automatic optimization techniques to search for the required parameters. The present authors have chosen not to do this for several reasons. Firstly, since data may be available for labelling, mitotic and cumulative labelling indices, the situation is rather different from that in which the parameters of a model are adjusted to fit a single set of data. The present model must fit several sets of data, and it would be invidious to ascribe weights to the various sums of squared residuals to obtain one statistic suitable in all instances. Expression (1) may not actually be minimized, therefore, for a given set of data. (In the case of a single FLM curve too a non-optimal set of estimates may be useful if a precise estimate of a particular parameter is desirable.)

This leads to the second reason for rejecting automatic techniques, namely that one of the

benefits of simulation is the insight it gives into the effects and interactions of the various parameters involved. This advantage is lost if the method is too computer-oriented; properly the computer is a tool in simulation studies and should be used in an interactive situation with the model builder in full control.

Thirdly, it is felt that automatic techniques involving sophisticated numerical optimization procedures may give a spurious illusion of accuracy. It is probable that any apparent improvement in estimation of parameters which an automatic method provides will be essentially model-dependent, reflecting, for example, the particular distributions chosen for phase times, rather than offering any further description of the system being modelled.

Finally it must be recognized that where a variable is capable of sudden alteration the technique of minimizing the residual sum of squares may be inappropriate. There is little justification for assuming that the indices follow a suitable distribution in the presence of considerable variation in rate of response between animals. It may be that fitting the curve by eye, however prone to subjective error, will yield better results than the use of standard methods, including transformation of data.

THE MATHEMATICS OF THE MODEL

I. *The system modelled*

(i) All cells are initially in a G_0 phase.
(ii) A proportion of the cells leave G_0 and pass through G_1, S, G_2 and M phases.
(iii) The rate at which these cells leave G_0 is a function of time only.
(iv) All cells take the same time to pass through a given phase.
(v) Progeny may leave the cycle (differentiate) or proceed through a further cycle.
(vi) The decycling probability at the end of each cycle may be constant, depend on the total number of cells in the cycle or on the number of cells which have left the cycle, or be any other function of time.

(vii) Labelling of cells in an S phase is assumed to be instantaneous. If this is not so, then estimates of the duration of $S(t_s)$ must be reduced and estimates of the duration of $G_1(t_{G1})$ increased by the duration of action of the labelling agent.

II. *Notation*

The relevant functions are:

$N(t)$	total number of cells in the system.
$I_L(t)$	proportion labelled of total.
$I_m(t)$	proportion in mitosis of total.
$I_p(t)$	growth fraction.
$FLM(t)$	proportion labelled of mitoses.
$I_{CL}(t)$	cumulative labelling index.
$\bar{\eta}_r(t)$	decycling probability after the rth cycle.

The following quantities are used in the analysis:

$D(t)$	number of cells which have left the system.
$S(t)$	number of cells in S phases.
$M(t)$	number of cells in M phases.
$G_0(t)$	number of cells in G_0.

$f(t)$	probability density function for cells leaving G_0.
k	number of cycles possible (for convenience of calculation only; k may be taken to be infinite in theory).
N_0	number of cells initially in G_0.
ρ	proportion of cells which leave G_0.
$L_{jr}(t)$	number of mitoses in rth cycle, labelled in jth cycle.
$L(t)$	number of labelled mitoses.
τ	time of labelling (FLM curve).
$\alpha_r^{(m)}$, $\omega_r^{(m)}$	time from leaving G_0 to start/finish of rth mitosis.
$\alpha_r^{(s)}$, $\omega_r^{(s)}$	time from leaving G_0 to start/finish of rth S phase.

(Thus $\omega_r^{(m)} - \alpha_r^{(m)} = t_m$ in the rth cycle;
$\omega_r^{(s)} - \alpha_r^{(s)} = t_s$ in the rth cycle;
$\omega_r^{(m)} - \omega_{r-1}^{(m)} = T_c$ for the rth cycle.)

For conciseness we define $P(\theta)$ by

$$P(\theta) = \rho N_0 2^{r-1} \prod_{j=0}^{r-1} (1 - \vec{\eta}_j(\theta + \omega_j^{(m)})); \; \omega_o^{(m)} = 0, \; \vec{\eta}_0 = 0;$$

so that, for example, $P(t - \alpha_r^{(s)}) f(t - \alpha_r^{(s)}) dt$ is the number of cells which start the rth S phase between times t and $t + dt$.

$$\tau_{1jr} = \tau + \alpha_r^{(m)} - \omega_j^{(s)}; \; \tau_{2jr} = \tau + \omega_r^{(m)} - \omega_j^{(s)};$$
$$\tau_{3jr} = \tau + \alpha_r^{(m)} - \alpha_j^{(s)}; \; \tau_{4jr} = \tau + \omega_r^{(m)} - \alpha_j^{(s)}.$$

III. *Solution*

We have the following relationships:

$$G_0(t) = N_0(1 - \rho \int_0^t f(\theta)\, d\theta) \tag{1}$$

$$D(t) = 2 \sum_{r=1}^{k} \int_{\omega_r^{(m)}}^{t} \vec{\eta}_r(\theta) P(\theta - \omega_r^{(m)}) f(\theta - \omega_r^{(m)})\, d\theta \tag{2}$$

$$N(t) = N_0 + \sum_{r=1}^{k} \int_{\omega_r^{(m)}}^{t} P(\theta - \omega_r^{(m)})\, f(\theta - \omega_r^{(m)})\, d\theta \tag{3}$$

$$S(t) = \sum_{r=1}^{k} \int_{t-\alpha_r^{(s)}}^{t-\omega_r^{(s)}} P(\theta)\, f(\theta)\, d\theta \tag{4}$$

$$M(t) = \sum_{r=1}^{k} \int_{t-\alpha_r^{(m)}}^{t-\omega_r^{(m)}} P(\theta)\, f(\theta)\, d\theta \tag{5}$$

Notice that since $\vec{\eta}_r(t)$ and hence $P(t)$ may depend on $D(t)$ or $N(t)$ equations (2) and (3) may be integral equations.

$$L_{jr}^{(t)} = \begin{cases} 0, & t < \tau_{1jr} \\ \int_0^{t-\alpha_r^{(m)}} P(\theta)\{f(\theta) - f(\tau - \omega_j^{(s)})\}\, d\theta, & \tau_{1jr} < t < \tau_{2jr} \\ \int_{t-\alpha_r^{(m)}}^{t-\omega_r^{(m)}} P(\theta)\, f(\theta)\, d\theta, & \tau_{2jr} < t < \tau_{3jr} \\ \int_0^{t-\omega_r^{(m)}} P(\theta)\{f(\tau - \alpha_j^{(s)}) - f(\theta)\}\, d\theta, & \tau_{3jr} < t < \tau_{4jr} \\ 0, & \tau_{4jr} < t \end{cases} \tag{6}$$

$$I_L(t) = \frac{S(t)}{N(t)} \qquad I_m(t) = \frac{M(t)}{N(t)}$$

$$I_p(t) = 1 - \frac{G_0(t - \alpha_1^{(s)} + \alpha_2^{(s)} - \omega_1^{(m)}) + D(t)}{N(t)}$$

$$I_{CL}^{(t)} = 1 - \frac{G_0(t - \alpha_1^{(s)})}{N(t)} \qquad L(t) = \sum_{r=1}^{k} \sum_{j=1}^{r} L_{jr}(t) \qquad \mathrm{FLM}(t) = \frac{L(t)}{M(t)}$$

THE COMPUTER MODEL

As indicated previously, when decycling probability is not a function of time alone but depends on another variable in the system, the mathematical formulation of the system involves an analytically intractable integral equation and step by step simulation is required. This must be carried out quickly as it will be used several times to investigate the behaviour or find the parameters of a particular system. Use is therefore made of the facts that the decycling probability changes slowly and that small changes in the decycling probability cause only small changes in the numbers of cells in any part of the system. This enables the value of $\bar{\eta}_r(t)$ to be approximated by assuming linearity over $t-2$, $t-1$ and t, and hence the functions $D(t), N(t), \mathrm{S}(t), \mathrm{M}(t)$ and $L(t)$ may be calculated. The estimates of the $\bar{\eta}_r(t)$ are then improved; no further iteration is required. $\mathrm{G}_0(t)$ may be calculated analytically; other integrals are evaluated using the trapezoidal rule, and economy is gained for $D(t)$, $N(t)$ and $\mathrm{S}(t)$ by using the values previously found for $t-1$. It is then simple to calculate the required indices.

The function $f(t)$ has been chosen to be an Erlangian distribution with parameter 2. The advantages of this distribution are that it is not an unreasonable approximation to the (unknown) distribution of times spent in G_0 after stimulation, and that it can be handled analytically; the program will accept other values of the parameter. Although the distribution may be integrated analytically, this is not important in the method of solution chosen, and an empirical distribution could be used, but it makes the solution for the case of constant decycling probabilities obtainable in an explicit form. An Erlangian distribution was preferred to a Gamma distribution not because an attempt can be made to justify it on the grounds of the 'method of stages' as Takahashi does (Takahashi, 1966) but because it is much faster to evaluate than the Gamma distribution.

The use of a multiparametric model may legitimately be criticized as an unprofitable technique if parameters are altered arbitrarily until the best possible fit has been obtained.

It was therefore thought advisable to restrict certain of the parameters of the model. If other good estimates of t_S or T_c are available it is not permissible to depart from these by more than a small amount; the time parameters for each cycle may conveniently be constrained, in the absence of any evidence to the contrary, to be the same, except for the first t_{G1}, which phase is experimentally indistinguishable from G_0. The decycling probability for each cycle will normally be the same and will only be taken to be dependent on the number of the differentiated cells if an adequate fit cannot be achieved while it remains constant. In this fashion the freedom of the model is constrained, and its ability to fit the several sets of data presented to it becomes the more impressive. Where data has been fitted nine parameters have been varied. In using the model for other purposes many more possibilities may be investigated.

RESULTS AND DISCUSSION

Fig. 2 shows the various functions which the model produces. For Figs. 2(a) and 2(b), the labelling and mitotic indices, the experimental data for the seminal vesicle is presented. This data has been fitted in the manner described.

Fig. 2(c) shows the curve which should be produced by an experiment which investigates the variation with time of the cumulative labelling index. The total cell numbers and the conventional growth fraction can also be examined. Because it is not possible to distinguish between cells in G_0 and G_1 the growth fraction of the model may be defined in several ways. We could consider all cells which have left G_0 and are still in the cycle to be proliferating, or we could exclude from this cells which have not yet reached the first S phase. In fact we have chosen an intermediate position, excluding only those cells which will not reach the first S phase in a time equal to the second G_1 phase. This is equivalent to an assumption of G_1s of equal length and G_0 a constant plus an Erlangian variate. The growth fraction curve given by the model is rather lower than is produced by the relationships $I_p = [I_{L\ (observed)}]/[I_{L\ (theoretical)}]$ when the theoretical labelling index is calculated either on the assumptions of exponential growth or steady state conditions, agreeing most closely during the early stages of proliferation, and less well as more and more cells fail to proliferate further. Figs. 2(d) and 2(e) are FLM curves, and 2(d) especially shows the dangers of using FLM techniques to estimate cell-cycle parameters if the growth fraction is not constant. The large influx of unlabelled cells reaching their first mitosis around 54 hr swamps the few labelled cells entering a second mitosis. It has been found that when labelling is more than about 60 hr after the initial stimulation of the system, modelled FLM curves bear little resemblance to real life in second and subsequent peaks, because for this aspect of the model variation in cell cycle parameters is no longer overshadowed so effectively by the variation in the length of the G_0 phase. Two such experiments are therefore convenient: one near the time that cells begin to arrive in S, and one at the peak of DNA synthetic activity.

Fig. 3 shows the labelling index and cumulative labelling index curves for the same system parameters as were used to fit the seminal vesicle data, except that the percentage of cells entering the cycle varies from 100% to 20% in steps of 20%. The same effect can be observed in the mitotic index.

Fig. 4 again uses the same parameters as Fig. 2, except that the decycling probability is kept constant, taking the values 0, 0·25, 0·5, 0·75 and 1. It is clear that the decycling probability has a great influence on the shape of the resulting curve, and it is likely that it can be estimated fairly closely.

Classical ideas of growth control such as the Weiss-Kavanu model propose a negative feedback mechanism from the functional or differentiated compartment acting upon the proliferative compartment to control the rate of cell production. This feedback may depend on the number of cells in the differentiated compartment or on some related variable. In the present experimental situation an induced peak in the proliferative indices occurs, followed

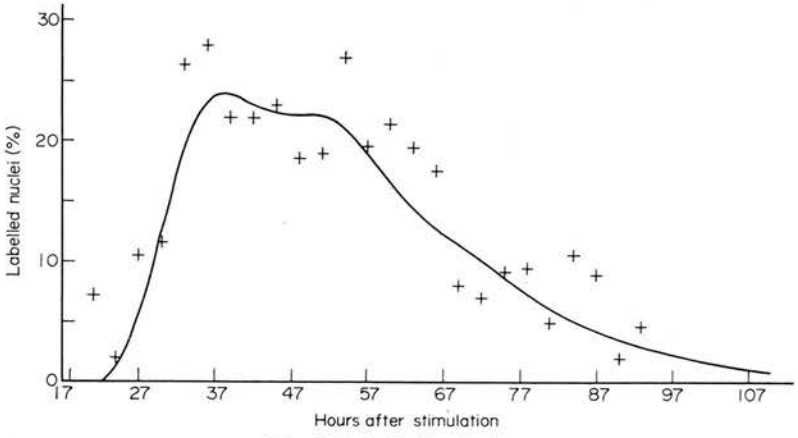

FIG. 2(a). Labelling index.

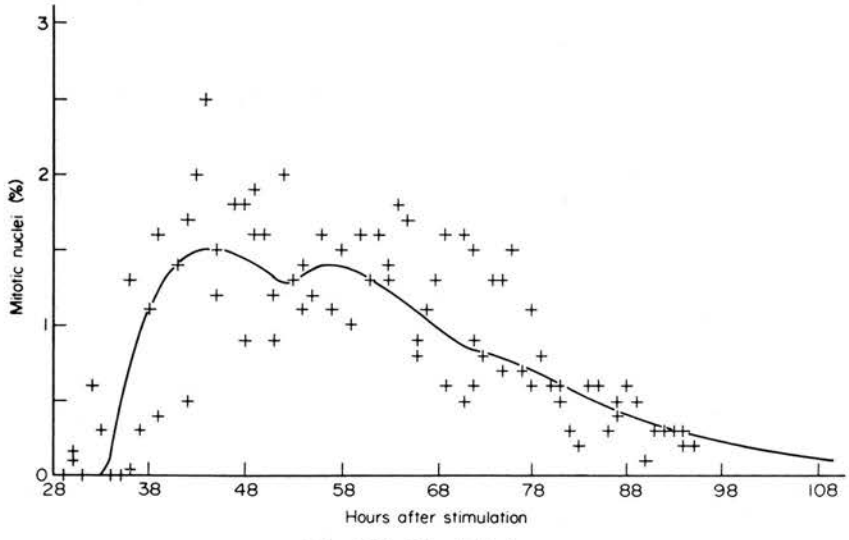

FIG. 2(b). Mitotic index.

FIG. 2. Output from the computer model with parameters chosen to fit data for the castrate mouse seminal vesicle. All cells leave G_0; mean time in G_0 is 28 hr; first G_1 phase is 22 hr. For all cycles after the first G_1, $t_s = 10$ hr, $t_m = 0{\cdot}7$ hr, $t_{G1} = 7$ hr, $t_{G2} = 1{\cdot}3$ hr. The decycling probability ($\vec{\eta}$) depends on the total number of cells (d) which have left the proliferative cycle through the relationship $= 0{\cdot}75\ (1 + 0{\cdot}001d)$.

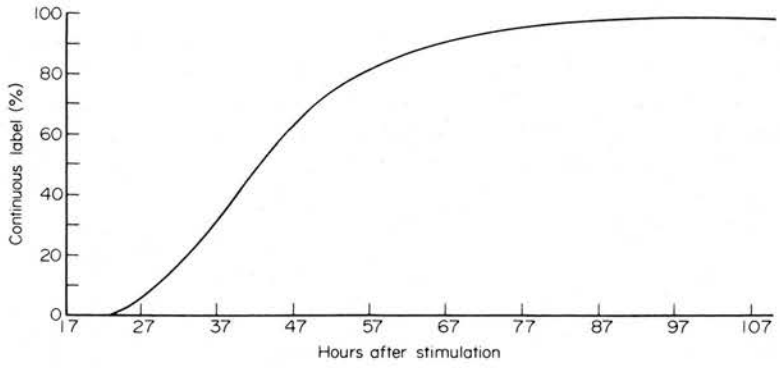

FIG. 2(c). Continuous labelling index.

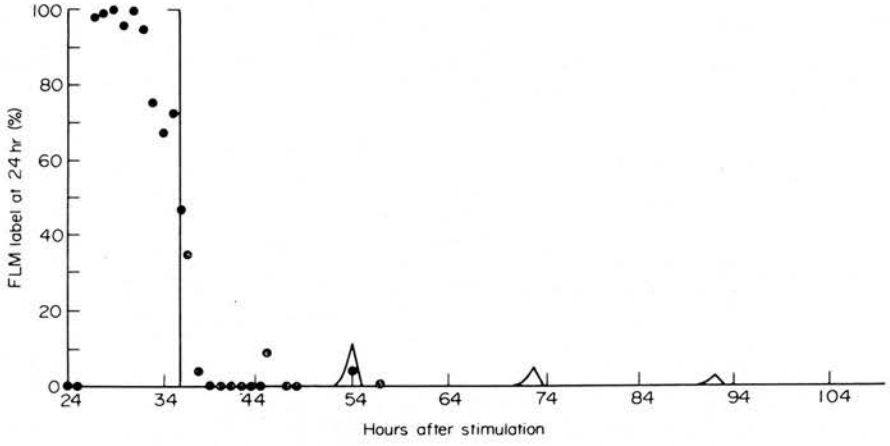

FIG. 2(d). FLM curve, label given at 24 hr after stimulation.

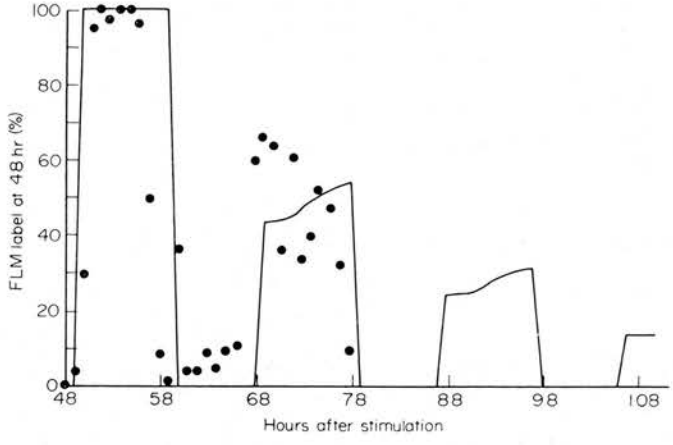

FIG. 2(e). FLM curve, label given at 48 hr after stimulation.

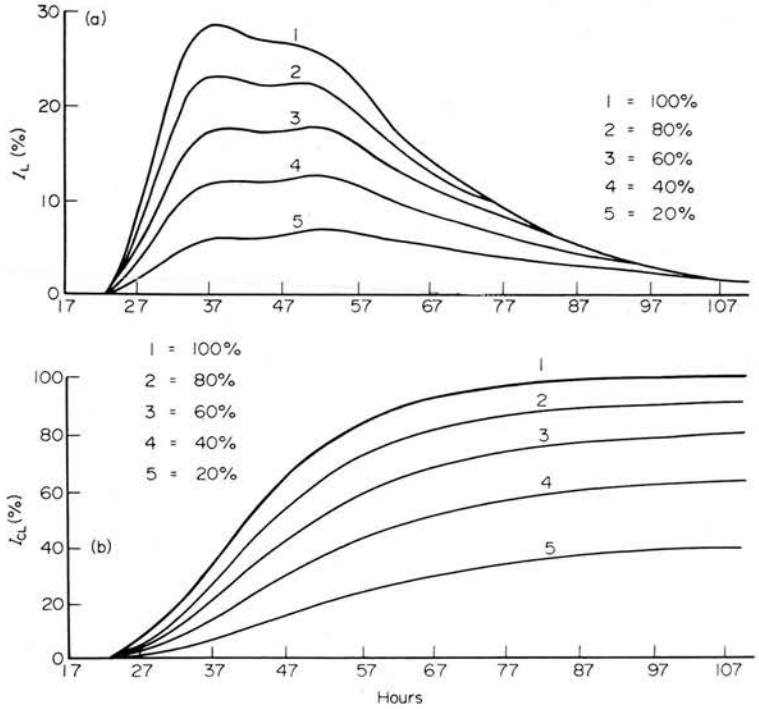

FIG. 3. The change in (a) labelling index and (b) continuous labelling index curves, for different percentages of cells leaving G_0. Other parameters are as in Fig. 2.

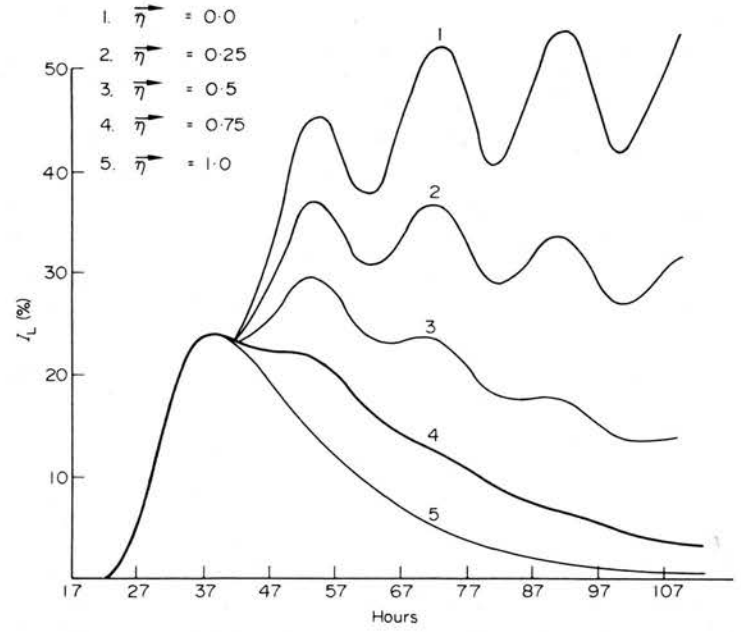

FIG. 4. The change in the labelling index curve for different decycling probabilities. Other parameters are as in Fig. 2.

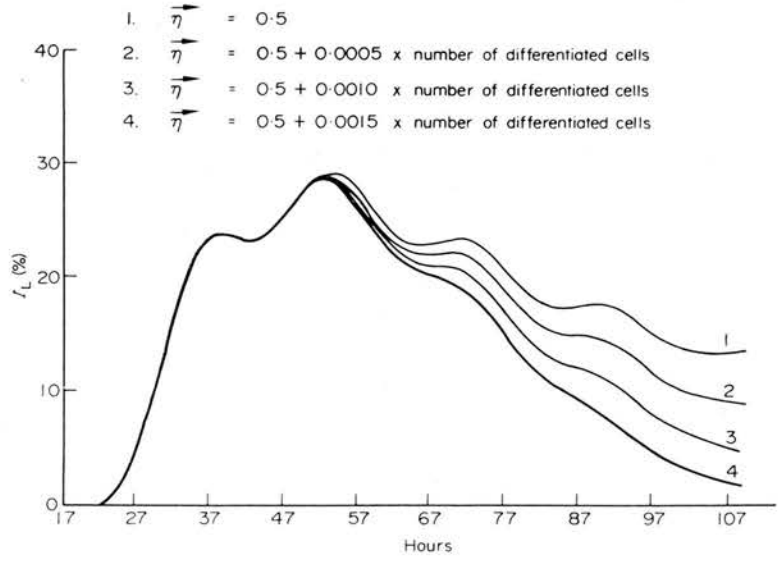

FIG. 5. The change in the labelling index curve for different relationships between the decycling probability and the number of differentiated cells. Other parameters are as in Fig. 2.

by a steady decrease despite the continuation of the stimulus. We consider that any form of feedback is likely to operate via the decycling probability. This concept has general application as similar results are obtained in uterine and vaginal epithelia in response to continuous oestrogen stimulation (Leroy, Galand & Chrétien, 1969). In Fig. 5 are shown I_L curves when $\vec{\eta}$ is made proportional to the number of cells in the differentiated compartment, with $\vec{\eta}$ changing at different rates. Such differences are reflected in the shapes of the curves. The factors determining the initial value of $\vec{\eta}$ are as yet obscure.

Alteration of the parameters of the model produces a wide range of response curve which at first sight seem qualitatively different. We suggest that the model described is applicable to many cases of induced DNA synthesis. This occurs through exogenous hormonal stimulation of a target organ in animals in which the source of trophic hormone has been removed, for example, stimulation of the uterus and vagina by administering oestrogen to castrated animals; and of adrenal cortex, thyroid gland and gonadal tissues deprived of pituitary trophic hormones. Further conditional renewal systems, such as are produced by partial hepatectomy or the induced proliferation of phytohaemagglutinin stimulated lymphocytes, may also be amenable to description by the model.

ACKNOWLEDGMENTS

The authors would like to thank their Heads of Department, Professor D. J. Newell and Professor A. G. Heppleston for their help and encouragement, and Dr G. Shearing for providing the Computek software; and also acknowledge the financial support of the North of England Branch of the British Empire Cancer Campaign.

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Reprinted from British Society for Developmental Biology Symposium,
The Cell Cycle in Development and Differentiation, edited by M. Balls and F. S. Billett
(Cambridge University Press 1973), pages 337–340.

TESTOSTERONE-INDUCED CELL PROLIFERATION AND DIFFERENTIATION IN THE MOUSE SEMINAL VESICLE. AN EXPERIMENTAL AND COMPUTER SIMULATION STUDY

59

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INTRODUCTION

The prostatic complex of the castrated male rodent responds to androgen stimulation by cell proliferation. The major features of this response have been described by Burkhardt (1940) and Allen (1958). Following androgen treatment there is a latent period of 15–20 h, then an increase in DNA synthesis followed by a wave of mitotic activity which reaches a peak at 40–50 h and thereafter declines to near-castrate levels. This response is typical of the models of induced DNA synthesis recently reviewed by Baserga (1971). Such experimental models are valuable because they allow examination of the events preceding cell division. These changes have been extensively reviewed by Cooper (1971). The cell cycle parameters of induced DNA synthesis in the castrate rodent prostate complex have been examined by Tuohimaa & Niemi (1968) who showed that the duration of DNA synthesis was considerably shortened after testosterone treatment. Morley & Wright (1972) have suggested that the fall in mitotic and labelling indices in the latter half of the proliferation response is a result of cells leaving the proliferative cycle. In the present work this concept is incorporated in a computer simulation model. The results of the simulation are consistent with the experimental data, and suggest further investigations to verify the theoretical model.

MATERIALS AND METHODS

Male Balb/c mice aged 3 months were castrated, and used two weeks later. Daily injections of 250 μg testosterone propionate in sesame oil were administered for up to 5 days. Mitotic indices (I_M) were based on a count of 2000 interphase cells. Labelling indices (I_L) were obtained in the same manner. Animals were killed 1 h after the intraperitoneal injection of

1 μCi/g of tritiated thymidine. Fraction labelled mitoses (FLM) curves were started 24, 48, and 72 h after the first injection of testosterone propionate.

A stochastic model incorporating the following features was developed for an IBM 360/67 computer: (1) The flow of cells from an undifferentiated or G_0 compartment followed an Erlangian distribution; (2) Cells passed through repeated cell cycles composed of G_1, S, G_2, and M compartments; (3) Following mitosis (M) a decycling probability was introduced determining the likelihood of daughter cells remaining in the proliferative compartment. The decycling probability could be fixed or made proportional to either the total number of cells, or the number of differentiated cells. Graphical print-out of simulated values of I_M, I_L (Figs. 1 and 2), growth fraction, FLM curves, I_L curves with continuous labelling and total cell number was obtained.

RESULTS

After a period of 20 h I_L increased to 25% at 35–45 h; and then gradually diminished to 5% by 90 h (see Fig. 1). The increase in I_M occurred later beginning at 30 h, and rising to 1.8% by 48 h. Thereafter I_M fell gradually to 0.4% at 90 h (see Fig. 2). FLM curves at 24, 48, and 72 h gave values of t_2 ($t_{G_2}+\frac{1}{2}t_M$) of 1.6, 2.0, and 1.8 h and t_S of 10.5, 8.0, and 8.0 h respectively. The duration of the cell cycle (T_c) was not measured by the FLM curve at 24 h since the no second peak was apparent, probably a result of dilution by unlabelled mitoses. T_c at 48 h was 17.5 h. Calculation of growth fraction at 48 h gave a value of 0.64. It was not possible to measure T_c from the FLM curve at 72 h, the absence of a second peak can be attributed to the small proportion of cells remaining in the proliferative cycle after 90 h. Since t_2 and t_S appear to remain largely constant throughout the proliferative response it is probable that the fall in I_L and I_M indicates a diminution in the number of cells entering DNA synthesis and mitosis.

The parameters obtained from FLM curves were incorporated in the computer simulation model. Constrained in this way the model produces values of I_M and I_L which are close to those obtained experimentally (continuous lines Figs. 1 and 2). The plateau seen in the experimental I_M and I_L curves is reproduced by the simulation model in which it is postulated that cells pass through successive waves of division (continuous line, Figs. 1 and 2).

The gradual fall in I_M and I_L in the model is a result of postulating that after mitosis each cell has a probability of leaving the proliferative cycle, and this probability rose from 0.75 to 1.0 as cells entered the differentiated compartment. This probability is the same as that described by Bresciani

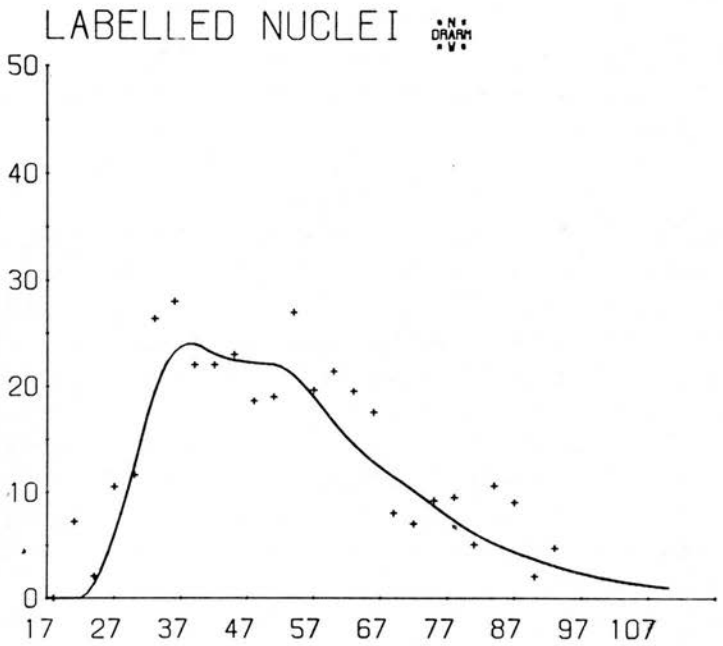

Fig. 1. Percentage labelled cells in the seminal vesicle of the castrate mouse during testosterone propionate treatment at 0, 24, 48, 72 and 96 h. +, experimental observations; −, computer simulation model.

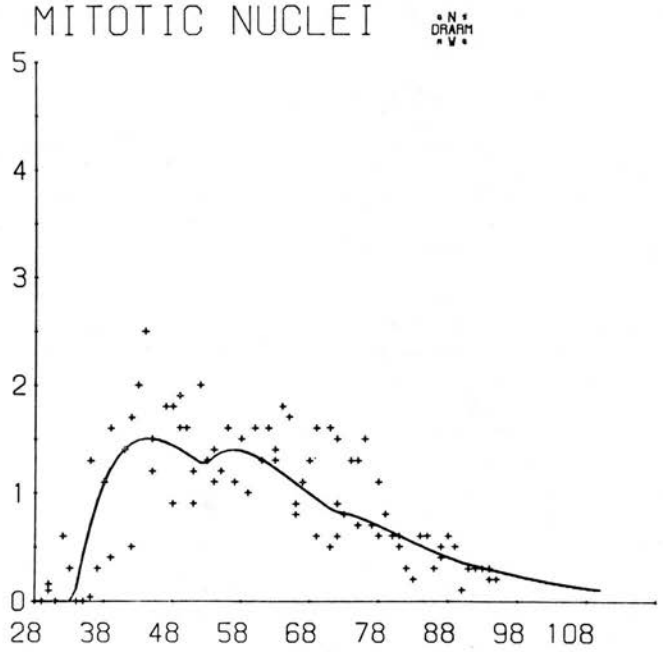

Fig. 2. Percentage mitotic cells in the seminal vesicle of the castrate mouse during testosterone propionate treatment at 0, 24, 48, 72 and 96 h. +, experimental observations; −, computer simulation model.

(1968) as $\vec{\eta}$. Similar values have been obtained in the testosterone-stimulated seminal vesicle using analysis of grain count distributions. (Morley & Wright, 1972).

DISCUSSION

The theoretical model described appears consistent with the experimental data, but contains an assumption that all cells in the G_0 compartment enter DNA synthesis in the proliferative response. It is important to know whether differentiation in the castrate seminal vesicle can occur without DNA synthesis and if there is a cell population unresponsive to testosterone stimulation. Continuous labelling with tritiated thymidine, or intermittent labelling at intervals less than t_S should label all cells entering DNA synthesis. Preliminary studies show that 100% of seminal vesicle cells enter the proliferative cycle. The theoretical model also predicts the increase in total cell number during proliferation. Experimentally, measurement of total DNA should reflect the increase in cell number.

Although the theoretical model described here is concerned with the seminal vesicle, preliminary results with the castrate mouse coagulating gland are encouraging. We believe that the model is flexible enough for use in the study of induced DNA synthesis in organs such as the partially hepatectomised liver. The computer model is rapid in operation and allows exploration of hypotheses which can be verified experimentally.

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David Appleton

A Cytokinetic Analysis of the Proliferative Response to Androgen in The Prostatic Complex of the Castrated Mouse

60

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Since the early work of Loewe & Voss (1930) many workers have confirmed the presence of a proliferative response in the prostatic complex of castrated rodents on androgen stimulation. The time-relationships of this response have also been analysed (Burkhardt, 1940; Allen, 1956, 1958; Coffey *et al.*, 1968; Ritter, 1969), and the results obtained have allowed Turkington (1971) to discern three components: a latent period of 15–20h, during which marked increases in the rates of protein and RNA synthesis occur (Kochakian, 1963), in preparation for the second component, a period of DNA synthesis and of cell division (Coffey *et al.*, 1968). During this second phase the thymidine-labelling and mitotic indices rise rapidly and reach a plateau at between 40 and 50 h after stimulation. Continuation of androgen administration results in a slow decline in mitotic index (Cavazos & Melampy, 1954) and in thymidine incorporation (Shepherd *et al.*, 1965).

At the same time as this induction of DNA synthesis there is evidence of cellular differentiation with hypertrophy, cytoplasmic vacuolation and production of secretion. The sequence described is akin to events following stimulation of DNA synthesis in the oestrogen-stimulated endometrium of the castrate (Epifanova, 1966), the partially hepatectomized liver (Grisham, 1962) and many other models found both *in vivo* and *in vitro* (Stein & Baserga, 1972).

There are several analogous events in all models of induced DNA synthesis. The first of these is the similar biochemical preparation for DNA synthesis, responsible for the common latent period of relatively constant duration. This finding indicates that before stimulation these cells are in a common proliferative state, which has been called the 'G_0' phase. With this statement an apparent anomaly becomes evident: the cells of the prostatic complex of the castrated animal and endometrium are non-functional, whereas hepatocytes are differentiated and fully functional. However, the latent periods between stimulation and the onset of DNA synthesis are remarkably similar in these systems, indicating that the state of differentiation is not a limiting factor.

G_0-phase cells are consequently in a phase of proliferative rest; it is difficult, of course, to distinguish between a true G_0 phase and a very long postmitotic interval (t_{g1}), but for practical purposes the distinction is immaterial. After castration the labelling and mitotic indices are even lower than in intact controls (Morehead & Morgan, 1967), and there is evidence of considerable cell loss; Kochakian (1963) found a 40% loss of ventral prostatic DNA within 14 days of castration.

Androgen stimulation of the prostatic complex of the castrated mouse thus provides an excellent model in which both the cytokinetic action of androgens and growth control during induced DNA synthesis may be studied. It has the additional and important advantage of easy reproducibility.

Despite an earlier attempt by Tuohimaa & Niemi (1968) to analyse cell proliferation after androgen stimulation, the underlying kinetic events responsible for the changes in the mitotic and labelling indices remain obscure. This applies particularly to the fall in

Table 1. *Proliferative indices and cell-cycle parameters in the seminal vesicle of the castrated mouse after androgen stimulation*

For explanation see the text.

Time after androgen stimulation (h)	t_2	t_{g1}	t_s	T_c	$I_{L(Exp.)}$	I_p†
24	1.6	—	10.5	101*	7.5	0.14
48	2.0	7.5	8.0	17.5, 25.7*	25	0.64
72	1.8	—	8.0	48.2*	12.5	0.32

* Calculated T_c assuming exponential growth.
† Assuming T_c to be 17.5h.

proliferative indices consequent on continuous testosterone stimulation. We have therefore undertaken a detailed study of the response, employing high-resolution radioautography with [^{3}H]thymidine.

Male Balb/c mice aged 3 months were castrated, and 14 days later were exposed to continuous androgen stimulation in the form of 250μg of testosterone propionate in oil given subcutaneously every 24h. In the seminal vesicle, after a latent period of 20h there was a rapid increase in the labelling index from less than 1% to over 25% at 35–45h; a steady fall to 5% at 90h then occurred. The mitotic index rose at about 30h, reaching a peak at 48h and then showing a similar decline. These time-relationships indicate the passage of a cohort of cells into DNA synthesis with subsequent mitosis. The temporal difference between the onset of DNA synthesis and mitosis gives an estimate of t_s+t_{g2} (the duration of DNA synthesis plus the duration of the premitotic interval) of 9.5h.

The parameters of the cell cycle were estimated by the FLM (fraction of labelled mitoses) technique, carried out at 24, 48 and 72h, and the results for the seminal vesicle are summarized in Table 1. At 24h it was not possible to obtain a second peak in the FLM curve, and consequently a direct measurement of T_c (the cell-cycle time) was not available. It is probable that the second division of labelled cells is swamped by the entry of large numbers of unlabelled cells into division. At 48h a distinct second peak was apparent, indicating that many of the labelled cohort had divided a second time, and giving a T_c value of 17.5h. At 72h, again no evidence of a second peak was obtained. However, no change in t_s was apparent, indicating that the decrease in the labelling index noted at this time was due to movement of cells out of the proliferative cycle.

Although a T_c value is only directly ascertainable at 48h, calculated values for T_c are given (Table 1), obtained by using Cleaver's (1967) equation and assuming exponential growth conditions, where the labelling index, I_L, is:

$$I_L = (\exp t_s \ln 2/T_c - 1)\cdot \exp t_2 \ln 2/T_c \qquad (1)$$

where $t_2 = (t_{g2}+\frac{1}{2}t_m)$. It is evident that the calculated values for T_c are long, and at 48h the calculated T_c is longer than the observed T_c. This latter observation indicates that throughout the response a proportion of the population is not in the proliferative cycle, i.e. a growth fraction exists and is less than unity. The only alternative is to postulate an extremely labile t_{g1}. We may calculate the growth fraction (I_p):

$$I_p = I_{L(Exp.)}/I_{L(Theor.)} \qquad (2)$$

where $I_{L(Exp.)}$ is the observed labelling index and $I_{L(Theor.)}$ is the theoretical labelling index calculated from eqn. (1) and assuming a growth fraction of unity. If we assume that the T_c value measured at 48h is applicable at 24 and 72h, then the increase and subsequent decrease of the labelling and mitotic indices may be explained by the changes in growth fraction (Table 1).

The proposal that changes in proliferating population are responsible for the variation

in proliferative indices is regarded as being much more tenable than postulating a very labile t_{g1}. Also, a marked change in t_{g1} without accompanying alteration in t_s (Table 1) is a most unusual situation. We may then assume a relatively constant T_c throughout the proliferative response; the rise in proliferative indices at the onset may be explained by non-functional cells moving into the proliferative cycle from G_0 phase. After each division a fraction of the resulting progeny move out of cycle with a consequent fall in growth fraction.

From these considerations a model of the kinetic events during the proliferative response can be set up; this is shown in Scheme 1. Cells leave G_0 phase and enter the first G_1-phase period, which appears to last about 20h. Most current opinion is for the siting after mitosis, and probably in late G_1 phase, of the decision whether to remain in the proliferative cycle or to 'decycle' and enter the differentiated compartment. The numbers of cells taking these alternative paths is controlled by the 'decycling probability'. This parameter is similar to that introduced by Bresciani (1968), who defined the distribution, or *d*-ratio, as the ratio of the proportion of cells re-entering the proliferative cycle ($\overleftarrow{\eta}$) to the proportion leaving it ($\overrightarrow{\eta}$); this latter concept is the decycling probability.

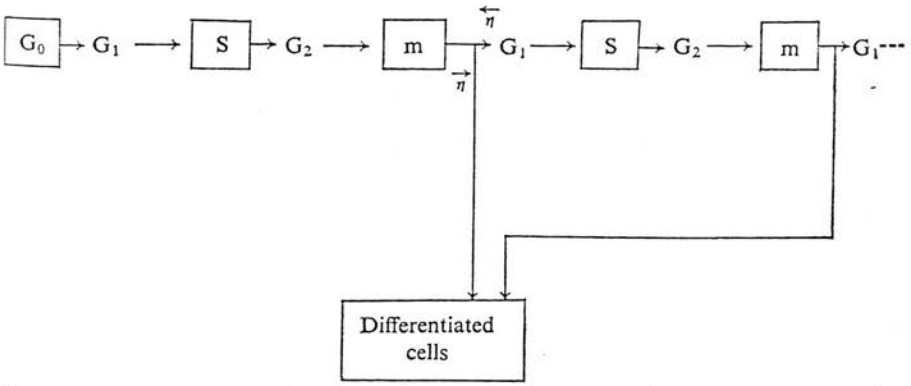
Studies involving the use of grain-count distributions (Morley & Wright, 1972) have shown that the decycling probability is close to 0.75. The decycling probability is central to a computer simulation that has been designed to model the events during the proliferative response. The model shown in Scheme 1 was treated analytically in an interactive environment involving graphical presentation of output. The curves were fitted to the experimental results by employing the transformation:

$$\sum_t v_t \cdot (\arcsin\sqrt{\lambda_t} - \arcsin\sqrt{\mu_t})^2 \qquad (3)$$

where λ_t is an experimental index (mitotic, labelling, FLM or continuous-labelling indices).

The integral equations of the system were set up and solved numerically. The model produced labelling and mitotic-index curves, and FLM curves that were consistent with the experimental findings for the seminal vesicle.

The coagulating gland was analysed experimentally in exactly the same manner; after a similar latent period a rise in proliferative indices was apparent, but in the case of the coagulating gland definite evidence of synchrony was obtained, with two definite peaks in the proliferative indices. Nevertheless it was possible to fit the experimental results with curves generated by the simulation. Further, the model produced continuous-labelling curves and curves describing cell population growth. These curves were



Scheme 1. *A model describing kinetic events in the castrated-mouse prostate complex after androgen challenge*

subsequently found to be consistent with experimental results available only after the computer print-out. The usefulness of the model has therefore been checked in prediction.

In conclusion, the proliferative events occurring in the mouse prostatic complex after castration and later rechallenge with androgen can be explained on the basis of a model involving movement into, and subsequently out of, a G_0 phase. Some indication of the rate of movement of cells out of G_0 phase is available from the labelling and mitotic-index curves; the rate of movement into G_0 phase and the role of cell loss in controlling the extent of the proliferative response are important aspects that require investigation.

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David Appleton

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CELL POPULATION GROWTH IN THE CASTRATE MOUSE PROSTATE COMPLEX: EXPERIMENTAL VERIFICATION OF COMPUTER SIMULATION

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(*Received* 14 *January* 1974; *revision received* 20 *February* 1974)

ABSTRACT

The stimulatory action of androgen on cell proliferation in the castrate mouse seminal vesicle and coagulating gland has been studied by DNA measurements in mice 14 days after castration. 100 hr after continuous androgen treatment the level of DNA had increased 2·5-fold in the seminal vesicle and coagulating gland compared with 14 days castrated controls. A mathematical model predicted this new experimental data when the parameters employed in the simulation were constrained by the results of previous experiments.

INTRODUCTION

Androgen, principally of testicular origin, maintains the adult dimensions and functional capacities of the accessory sex glands, while castration induces involution of the glands. DNA, RNA and protein levels in the glands decline for approximately 14 days after castration. The fall in DNA content of these organs indicates that an appreciable degree of cell loss occurs as shown by the ultrastructural studies of Kerr & Searle (1973) and Helminen & Ericsson (1972).

Turkington (1971) discerned three components in the proliferative response in the prostate complex of the castrate rodent upon androgen stimulation; a latent period of between 15 and 20 hr in which changes associated with the onset of DNA synthesis took place (Kockakian, 1963; Ritter, 1969), a second period where a rapid rise in the level of DNA synthesis (Coffey, Shimazaki & Williams-Ashman, 1968; Ritter, 1969) is closely followed by a rapid rise in the rate of cell division (Burkhardt, 1940; Allen, 1956, 1958), and finally a period in which both the labelling and mitotic indices decline despite continued androgen treatment (Shepherd *et al.*, 1965; Morley, Wright & Appleton, 1973).

The cytometric studies of Cavazos & Melampy (1954) and Ritter (1969) have provided evidence of cellular differentiation by hypertrophy, vacuolation and the production of secretion during the proliferative response; consequently this is an example of induced DNA

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synthesis similar to that occurring in the oestrogen-stimulated uterus (Finn, Martin & Carter, 1969) and the isoproterenol-stimulated submandibular gland of the rat (Barka, Chang & Van der Noen, 1973). In all models of induced DNA synthesis, a latent period is observed before cells enter DNA synthesis (Stein & Baserga, 1972), suggesting that before stimulation the sensitive cells are in a resting state, which has been called the G_0 state (Epifanova & Terskikh, 1969; Morley *et al.*, 1973).

Morley *et al.* (1973) showed that cell cycle parameters remained effectively constant throughout the duration of the proliferative response in the castrate mouse seminal vesicle. They concluded that the decline in the labelling and mitotic indices was due to a movement of cells out of the proliferative cycle during the later stages, and devised a model of the cell kinetics incorporating the concept of a decycling probability ($\bar{\eta}$); a parameter which determines the proportion of newborn progeny that move out of the proliferative cycle. The model has been treated analytically in an interactive environment with a computer (Appleton, Morley & Wright, 1973; Morley *et al.*, 1973).

This communication tests the validity of the model by comparing the cell population growth described by simulation in the castrate mouse prostatic complex during the induced proliferative response with cell population growth estimated by DNA measurements.

MATERIALS AND METHODS

Male Balb/c mice aged 3 months were castrated. Fourteen days later between 09.00 and 10.00 hours each animal was injected subcutaneously with 250 μg of testosterone propionate (Koch-Light) dissolved in sesame oil. Thereafter testosterone propionate was injected every 24 hr until the animal was killed.

Animals were killed by cervical dislocation at intervals of 3 hr for up to 100 hr, after the initial injection of testosterone propionate; the glands were removed and stored in liquid nitrogen until DNA estimation.

The DNA was extracted from the tissues by a modification of the procedure of Schneider (1945). Homogenates (10% w/v) were prepared at 4°C in 0·1 M Tris-EDTA buffer, pH 8·0, using a mechanically driven ground glass homogenizer. The nucleic acids, together with proteins and other large molecules were precipitated from the homogenates by the addition of excess ice cold 10% trichloroacetic acid (TCA). In accordance with Munro & Fleck (1966) lipid extraction was found not to be necessary, and DNA was extracted from the tissues by treating the TCA precipitates for 30 min with 2% perchloric acid at 90°C. DNA was measured in the hot acid extract by the modified diphenylamine reaction described by Burton (1968).

RESULTS

The change in the amount of diphenylamine detectable DNA with time after the first injection of testosterone propionate is shown in Fig. 1 for the seminal vesicle and Fig. 2 for the coagulating gland.

In the conversion of DNA estimations to actual cell number it is necessary to estimate, and subsequently delete the DNA responsible for excessive ploidy above the diploid amount in any of the cells. Labelling indices are available throughout the period of stimulated cell proliferation (see Figs. 1 and 2, Morley *et al.*, 1973) and assuming the mean ploidy of labelled

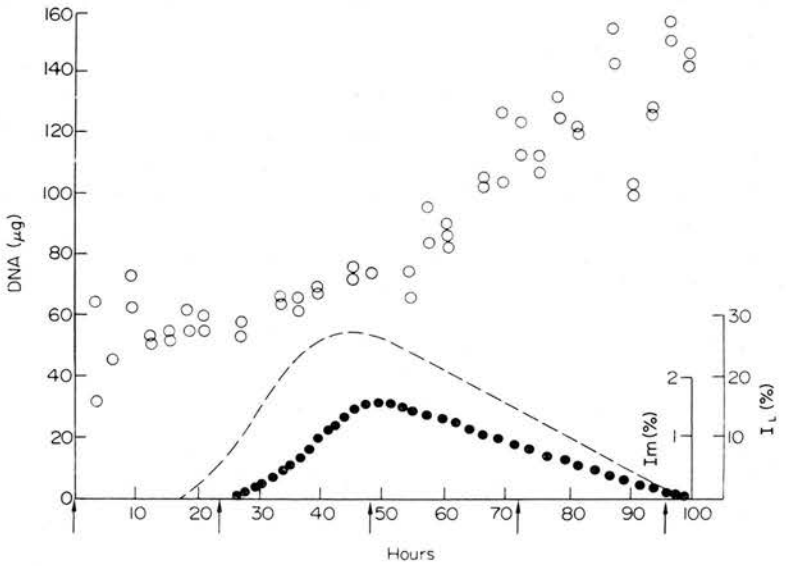

FIG. 1. Open circles: DNA in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate. Arrows show times of subcutaneous injections. Below: Labelling index (dashed line) and mitotic index (solid circles) in the seminal vesicle during the proliferative response.

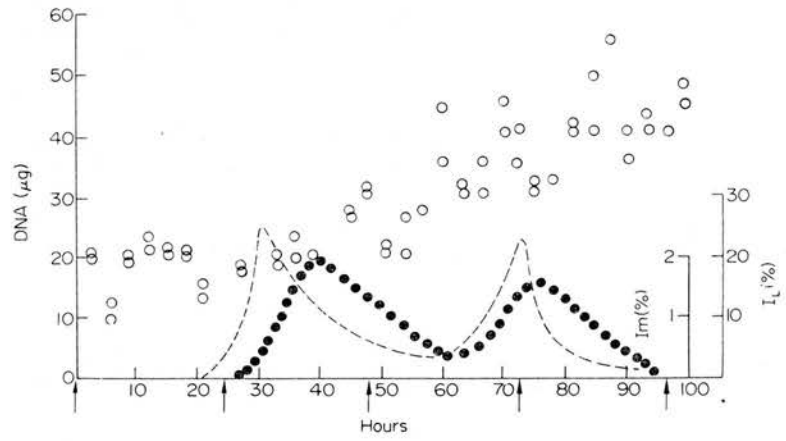

FIG. 2. Open circles: DNA in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate. Arrows indicate times of subcutaneous injections. Below: Labelling index (dashed line) and mitotic index (solid circles) in the coagulating gland during the proliferative response.

cells is 3N, subtracting one-third of the DNA from the labelled population will produce a new estimate of DNA which is a more accurate indication of the number of diploid cells. Similarly, cells in the post-mitotic interval and mitosis with a 4N ploidy will cause an overestimate of cell number from the measured DNA. The size of the 4N cohort was estimated

from the relative phase durations (see Table 1) calculated by Morley *et al.* (1973). The 4N cohort was calculated to be 27·5% as large as the population in DNA synthesis in the seminal vesicle and 55·6% as large as the DNA synthesizing population in the coagulating gland. Deleting half the DNA from the 4N population corrected for the excessive ploidy. All such corrections for excessive ploidy are approximate, and a number of assumptions have to be made. Firstly, cells enter DNA synthesis asynchronously, establishing a mean ploidy of 3N amongst the thymidine labelled population. This is probably incorrect even if entry into DNA synthesis is random; there is evidence that DNA synthesis does not occur evenly throughout the synthetic phase (Wright, 1971). Secondly, in the estimation of the size of the 4N population from the phase durations it is assumed that there is a rectangular age-distribution of cells throughout the cycle.

TABLE 1. Duration in hours of cell cycle parameters in the seminal vesicle and coagulating gland of castrate male Balb/c mice after androgen stimulation (Morley *et al.*, 1973)

	Cell cycle parameter		
Target organ	t_s	t_{G2}	t_m
Seminal vesicle	8	1·6	0·6
Coagulating gland	9	4·0	1·0

From the adjusted measurements of DNA the number of cells was calculated assuming that 6·2 pg of DNA was present in each nucleus. This amount is found in the nuclei of most organs and tissues of rats, whatever their age (Enesco, 1957). The mean value of the population estimates made in the first 15 hr was taken to be the initial size of each gland population before stimulation. All estimates made subsequently were expressed as percentages of the initial population.

The population growth occurring with time after the first injection of testosterone propionate is shown in Fig. 3 (open circles) for the seminal vesicle and Fig. 4 for the coagulating

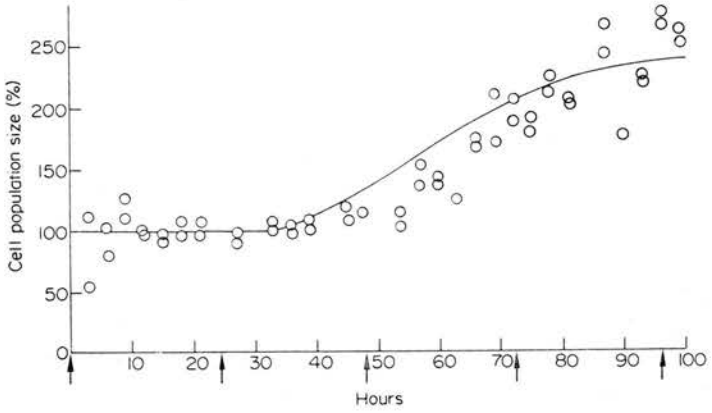

FIG. 3. Open circles: Cell population growth in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). Continuous line: population growth in the seminal vesicle indicated by the model.

gland. The population growth described by the model is shown by a continuous line in Figs. 3 and 4 for the seminal vesicle and coagulating gland respectively. The parameters used in simulation were constrained by previous experiments (Morley *et al.*, 1973). The model prescribed that 95% of cells in the seminal vesicle and 85% of cells in the coagulating gland were responsive to androgen, this being established experimentally by a continuous labelling technique with tritiated thymidine (Alison *et al.*, unpublished results).

After a quiescent period of between 30 and 40 hr, population growth occurs rapidly during the next 40–50 hr before declining in the latter part of the response. The cell population increased by a factor of 2·5 over 100 hr in both glands, and the rate of growth was very similar to that indicated in simulation.

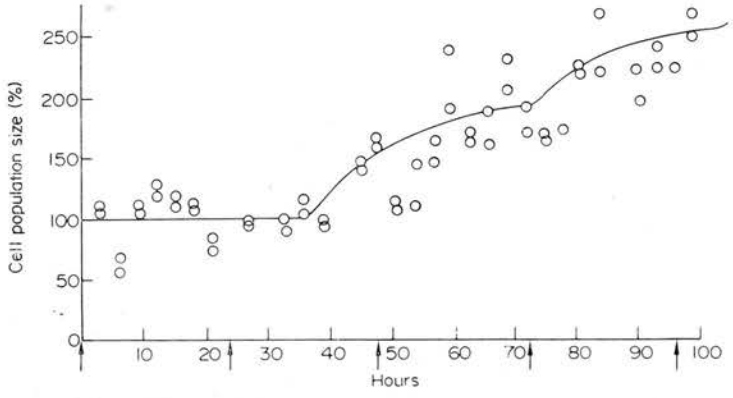

FIG. 4. Open circles: Cell population growth in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). Continuous line: population growth in the coagulating gland indicated by the model.

DISCUSSION

A model, like any other hypothesis, must be tested by its usefulness in prediction. In this communication the model, constrained by cell cycle parameters, has been tested by independent data in the form of DNA measurements and has proved to be consistent in prediction.

The method employed for compensating for the variation in ploidy may be criticized. Enesco & Leblond (1962), however, propose that satisfactory figures for cell number could be calculated from the DNA content of proliferating tissues, if it (i.e. the measurement of DNA content) is considered as an estimate of the number of cells that would have been present a short period after sacrifice. Neither method we suspect is very accurate, but as the DNA involved in compensating for excessive ploidy was usually less than 10% of the measured amount, a high degree of accuracy from the method employed was considered not to be necessary in this study.

The slight divergence of the fitted line to the data for both the seminal vesicle and coagulating gland, particularly during the early stages of population increase, may indeed be due in part to anomalies in correction.

Previously, studies involving the actions of exogenous androgens upon the prostatic organs of castrated rodents have been primarily interested only in the magnitude of the proliferative response (Kochakian, 1963; Tuohimaa, Oksanen & Niemi, 1973). Hitherto

the detailed analysis of the rise in cell population itself has been ignored, and this study has attempted to draw conclusions from the detailed analysis of that rise.

ACKNOWLEDGMENTS

We are grateful to Professor A. G. Heppleston for continued encouragement and advice. Miss E. Robinson rendered skilled technical assistance. We gratefully acknowledge the financial support of the North of England Branch of the Cancer Campaign.

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© *Journal of Microscopy, Vol.* 106, *Pt* 2, *March* 1976, *pp.* 221–237.
Received 15 *July* 1975; *revision received* 14 *November* 1975

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Cell proliferation in the prostate complex of the castrate mouse

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SUMMARY

Cell proliferation during 100 h of continuous androgen challenge was studied in the seminal vesicle and coagulating gland of Balb/c mice castrated 3 days or 14 days prior to the first daily injection of 250 μg testosterone propionate. Continuous labelling with [^{3}H] thymidine indicated that the seminal vesicle was almost totally responsive to androgen, as early as 3 days after castration, whereas the androgen sensitivity of the coagulating gland increased from 30% at 3 days after castration to 85% at 14 days after castration. In both tissues the magnitude of the proliferative reaction could be related to the extent of cell loss prior to stimulation. The duration of the pre-replicative phase in the response of the seminal vesicle to androgen was 20–25 h both at 3 and 14 days after castration. In the coagulating gland the pre-replicative phase was 40 h at 3 days after castration and 20 h at 14 days after castration.

The maximum uptake of [7α-^{3}H] testosterone administered to mice 3 days after castration was significantly greater ($P < 0{\cdot}01$) in the seminal vesicle compared to the coagulating gland. At 14 days the seminal vesicle and coagulating gland exhibited a similar capacity for uptake.

The *in vivo* metabolism of [7α-^{3}H] testosterone was studied by thin layer chromatography 30 min and 120 min after administration. A high proportion of the radioactivity extracted from all the tissues was associated with highly polar steroids. At 3 days after castration, the seminal vesicle, 2 h after administration of radioactive testosterone, retained a much higher proportion of radioactivity associated with dihydrotestosterone than did the coagulating gland.

The localization of steroid in mice 3 days after castration was studied by dry-mount autoradiography at intervals up to 2 h after the injection of [1,2,6,7(n)-^{3}H]-testosterone. A heavier deposition of silver grains was observed over autoradiographs of the seminal vesicle. In the seminal vesicle the grains were primarily located over nuclear areas whereas in the coagulating gland the grains were diffusely distributed over both nuclear areas and over cytoplasmic areas.

INTRODUCTION

The production of androgens in the testis is essential to maintain the functional capacities of the accessory sex organs of reproduction. The withdrawal of andro-

genic steroids causes the accessory organs to undergo a gradual regressive change, occurring over 2 weeks.

The processes involved in the atrophy of the accessory organs have received much attention. Organ weight declines rapidly following castration, with a loss of between 60% to 85% after 2 weeks (Kochakian, 1963; Tuohimaa *et al.*, 1973). The decline in DNA content is not as rapid, with losses of 40%, 2 weeks after castration in the mouse prostate (Kochakian & Harrison, 1962), 50% in the mouse coagulating gland (Alison *et al.*, 1974a) and 65%–70% in the mouse seminal vesicle (Alison *et al.*, 1974b). The reduced amount of DNA indicates a considerable degree of cell loss from these organs, as shown by an 85% reduction in the number of nuclei isolated from the rat prostate 14 days after castration (Lesser & Bruchovsky, 1973). Ultrastructural studies also indicate considerable cell loss; Kerr & Searle (1973) have noted the depletion of cells by apoptosis in the involution of the rat prostate after castration.

The injection of androgen into castrated rodents results in cell proliferation in the accessory organs, with a rapid restoration of tissue weight and a resumption of secretory activity.

The first biochemical manifestation of androgen action is a stimulation of RNA synthesis (Wicks & Kenny, 1964). The induction of DNA synthesis by androgens is preceded by a quiescent period of between 15–25 h, and as in other models of induced DNA synthesis this time lag has become known as the *pre-replicative period* (Stein & Baserga, 1972). The regulating events necessary for the onset of DNA synthesis, namely RNA and protein synthesis (Cooper, 1971) would seem to take place during this pre-replicative period.

The end of the pre-replicative period is marked by a sudden burst of DNA synthesis in the accessory organs, with a subsequent sharp rise in mitotic activity. Both labelling and mitotic indices reach a peak between the second and fourth days of hormone treatment, and thereafter steadily decline despite continued hormone administration (Shepherd *et al.*, 1965; Coffey *et al.*, 1968; Morley *et al.*, 1973). Morley *et al.* (1973) considered that the induced wave of proliferation was caused by a changing proportion of the cell population traversing the proliferative cycle during the response. The restoration of the cell population to the complement found in the accessory organs of intact animals has usually been achieved within 4–6 days of continued hormone treatment (Lesser & Bruchovsky, 1973), whilst restoration of tissue weight occurred at an appreciably slower rate, taking as long as 2 weeks (Kochakian, 1963; Parrot, 1974). This disparity suggests that the response to androgen challenge is made up of two components, cell multiplication and subsequent cell differentiation with concomitant return of secretory activity. Initially the response is one of cell multiplication which latterly gives way to one of cell differentiation, and we have suggested (Morley *et al.*, 1973) that the size of such a non-cycling compartment may be responsible for the curtailment of cell proliferation. A computer simulation of this hypothesis has proved to be consistent with experimental findings (Appleton *et al.*, 1973).

The increased binding of radioactive androgens in accessory reproductive organs after castration is well known (Mainwaring, 1969; Tveter, 1969; Fang *et al.*, 1969). In such androgen dependent tissues the naturally secreted hormone, testosterone, undergoes extensive metabolism for the complete biological expression of its action (Bruchovsky & Wilson, 1968; Robel *et al.*, 1971).

Most studies of the metabolism and proliferative action of androgens on target prostatic tissue have taken little account of the protracted delay between castration and the administration of androgen. The present study was undertaken to investigate whether the uptake and metabolism of testosterone and the ensuing prolifera-

tive reaction in the seminal vesicle and coagulating gland is dependent upon time elapsed since castration.

MATERIALS AND METHODS

Male Balb/c mice were castrated by an abdominal approach at 3 months of age. The regressive changes occurring after castration were monitored. All further studies were designed to elucidate the growth characteristics of androgen induced regeneration in the seminal vesicle and coagulating gland of the castrate mouse, when the duration between castration and the onset of androgen treatment was either 3 or 14 days. Each animal was injected subcutaneously with 250 μg of testosterone propionate (Sigma Chemical Co. Ltd) dissolved in sesame oil between 09.00 and 10.00 h. Thereafter testosterone was injected every 24 h until the animal was killed.

Determination of DNA and RNA

The method of extraction of nucleic acids from the tissues was essentially that described by Schnieder (1945). RNA was assayed by the orcinol reaction using calf liver RNA (Sigma) as standard, and DNA by the modified diphenylamine reaction using acetaldehyde, described by Burton (1968), with deoxyribose as standard.

Determination of thymidine labelling (I_s) and mitotic (I_m) indices

[6-^{3}H] Thymidine (5·0 Ci/mM) was obtained from the Radiochemical Centre, Amersham, Bucks.

When tritiated thymidine is made available to a population of proliferating cells it is incorporated by the cohort in the DNA synthetic phase (S). The labelling index can be used as a guide to the proliferative activity of the cell population.

Values of I_s and I_m were obtained through the period of the stimulated cell proliferation as described previously (Morley *et al.*, 1973).

Continuous labelling

Animals were given tritiated thymidine in a dosage of 0·5 μCi/g body weight throughout the period of stimulation at intervals of 4 h; one animal was killed 1 h after each injection. A 4 h interval betwen injections of tritiated thymidine was considered sufficiently short to label all cells synthesizing DNA (the DNA synthetic phase was found to be 8–10 h in the seminal vesicle, Morley *et al.*, 1973). The system under study has been successfully simulated analytically by a mathematical model (Appleton *et al.*, 1973). To estimate the responsive proportion of the cell population multiple simulations were made by altering the percentage of cells which were responsive, until a good fit was obtained to the experimental points.

Cell cycle parameters

The cell cycle parameters were estimated by the fraction labelled mitoses method (FLM) (Quastler & Sherman, 1959). The FLM studies were begun 48 h after the first testosterone injection because it had been found that the system was subject to the least alteration in growth fraction at this time (Morley *et al.*, 1973).

Testosterone uptake

[7α-^{3}H]Testosterone (10·7 Ci/mM) was obtained from the Radiochemical Centre, Amersham, Bucks. On the day of the experiment a portion of the ethanol stock

solution was evaporated to dryness and dissolved in saline. Each animal was given 18·5 μCi (about 0·5 μg) of [7α-^{3}H]testosterone by i.v. injection using a micrometer syringe. Organs were removed at intervals for up to 2 h, placed in counting vials and digested with 1 ml hyamine hydroxide (Koch-Light). Radioactivity was counted in a L.K.B. Wallac Liquid Scintillation Counter after the addition of 10 ml toluene containing PPO (5 g/l) and POPOP (0·075 g/l). Counts were converted to disintegrations per min (d.p.m.) using an external gamma radiation source.

Testosterone metabolism

In these experiments [7α-^{3}H]testosterone was given by the route and dosage described above. Animals were killed 0·5 h and 2 h after injection. The tissues from ten to eighteen animals were pooled for each analysis. The distribution of radioactivity in the various non-polar transformation products of testosterone was investigated by thin layer chromatography using a solvent system of chloroform and methanol; 98·25 : 1·75 (v/v). Reference steroids were visualized by exposure to iodine vapour.

Dry-mount autoradiography

Animals were given 200 μCi (about 0·64 μg) of [1,2,6,7(n)-^{3}H]testosterone by i.v. injection. Animals were killed at intervals for up to 2 h, tissues rapidly removed and immersed in liquid nitrogen. Sections of 6 μm thickness were cut in a cryostat at approximately -20°C and placed in contact with coverslips precoated with Kodak A.R. 10 stripping film. The sections were exposed for 7 days at -20°C and films developed in Kodak D19b.

RESULTS

The effect of castration on tissue weight is shown in Fig. 1. Weight falls steadily for 10 days and then is maintained at a low level, 16%–20% of normal.

The effect of castration on DNA content is shown in Fig. 2. Castration has an almost immediate effect on the DNA content in the seminal vesicle. By 72 h the DNA content has been halved, and at 7 days only 33% of the normal complement remains; thereafter little further change occurs. In contrast, 80% of the coagulating gland DNA is retained at 72 h after castration, and at 14 days the DNA content is 53% of the normal value.

The effect of the testosterone propionate on RNA in the castrate mouse seminal vesicle is shown in Fig. 3. At 3 days after castration (Fig. 3a) there is no evidence of an increase in RNA for up to 50 h after the first injection. At 100 h values are 1·5–2 times greater than normal. At 14 days after castration (Fig. 3b) an increase in RNA is apparent 30 h after the first injection, with the normal complement attained by 100 h.

The effect of testosterone propionate on RNA in the castrate mouse coagulating gland is shown in Fig. 4. At 3 days after castration (Fig. 4a) RNA falls slowly for 30 h after the first injection. Thereafter RNA increases, normal values being attained by 60 h. At 14 days after castration (Fig. 4b) RNA increases rapidly 15 h after the first injection, and the normal complement is essentially restored by 50–60 h.

The effect of testosterone propionate on DNA in the castrate mouse seminal vesicle is shown in Fig. 5. At 3 days after castration (Fig. 5a) the DNA complement is reduced to 50% of normal values, and no increase is observed until 40–50 h after the first injection. The normal complement is restored by 100 h. At 14 days after castration (Fig. 5b) the DNA complement is only 30% of the normal value. An increase in DNA is apparent 30–40 h after the first injection, and by 100 h the DNA content is almost restored to the normal value.

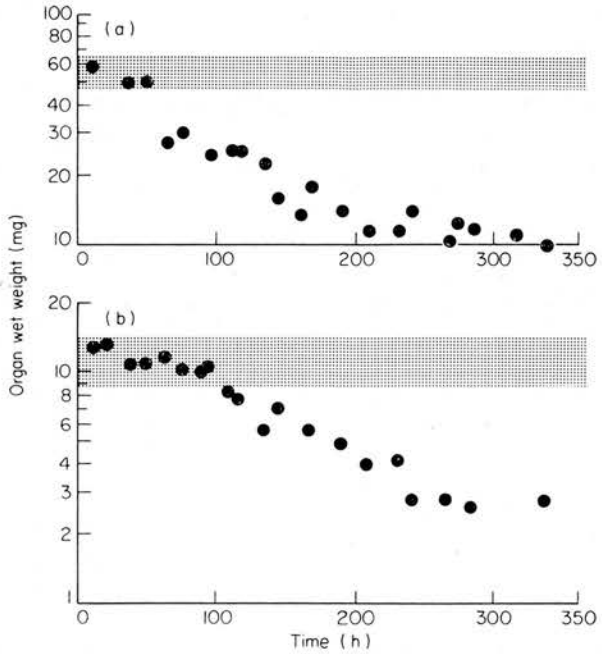

Fig. 1. Seminal vesicle wet weight (a) and coagulating gland wet weight (b) after castration. Three month old Balb/c mice were castrated at time 0. The upper limit of the shaded area represents the mean weight in intact mice (n = 18), the bottom limit represents the lower 95% confidence limit.

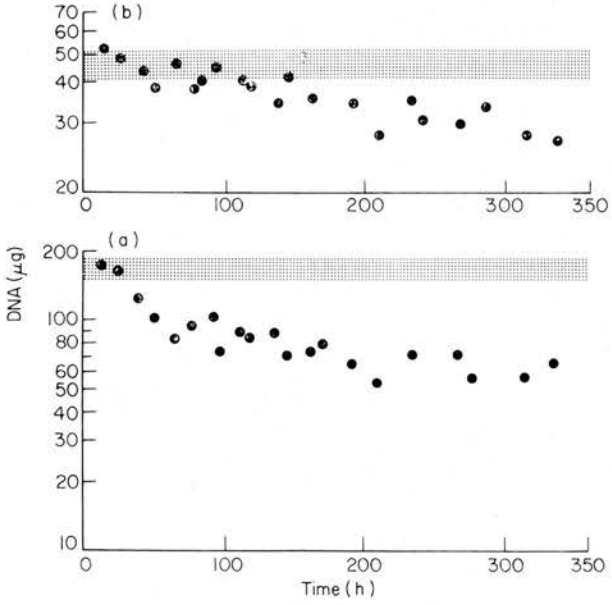

Fig. 2. Seminal vesicle DNA (a) and coagulating gland DNA (b) after castration. The upper limit of the shaded area represents the mean DNA content in intact mice (n = 18), the bottom limit represents the lower 95% confidence limit.

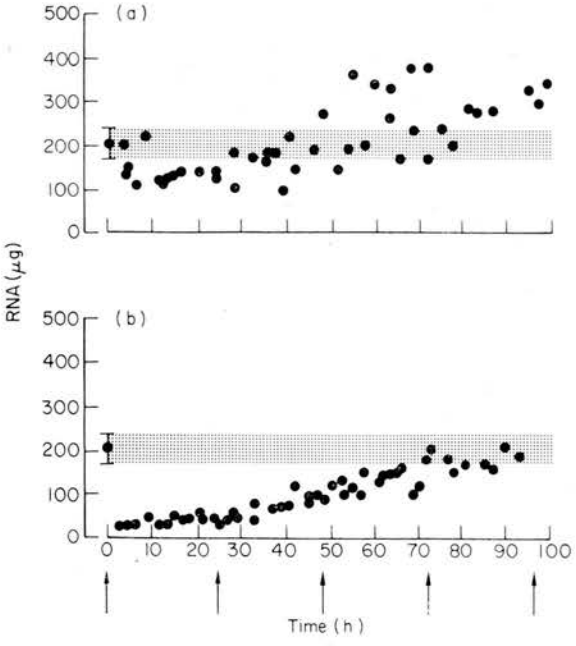

Fig. 3. RNA in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b). Shaded area represents mean $\pm$s.d. in intact mice (n = 18).

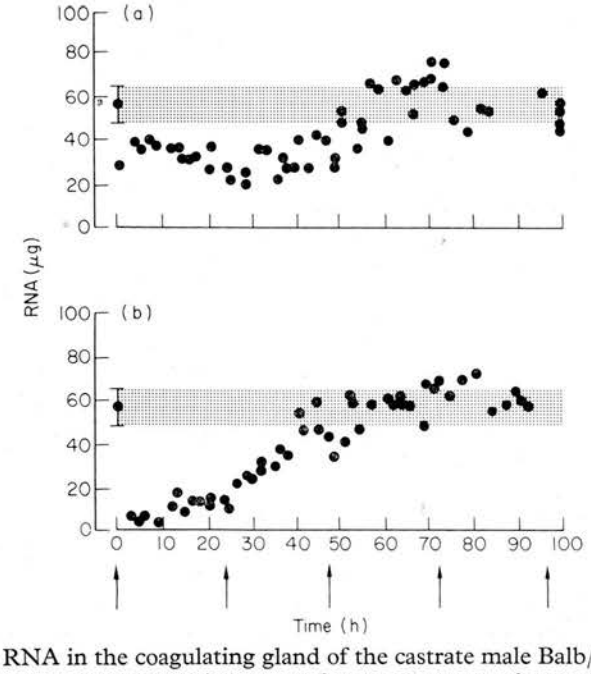

Fig. 4. RNA in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b). Shaded area represents mean $\pm$s.d. in intact mice (n = 18).

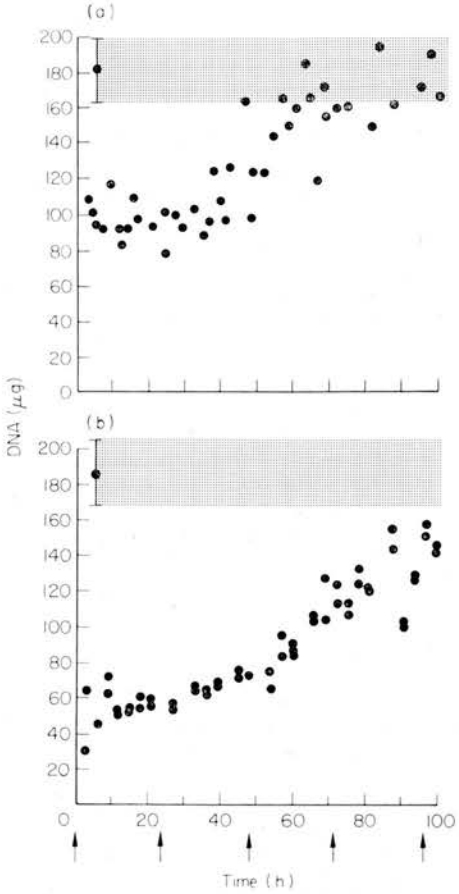

Fig. 5. DNA in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b). Shaded area represents mean $\pm$s.d. in intact mice (n = 18).

The effect of testosterone propionate on DNA in the castrate mouse coagulating gland is shown in Fig. 6. At 3 days after castration (Fig. 6a) the DNA content is variable, being at most only 20% below normal values. A small increase in DNA is observed at 60–70 h after the first injection. At 14 days after castration (Fig. 6b) the DNA content is only 40% of normal values. An increase is apparent 40–50 h after the first injection, resulting in restoration of the normal complement by 100 h.

The variation of 1_s and 1_m in the seminal vesicle with time after androgen administration is shown in Fig. 7. At 3 days after castration (Fig. 7a) there is a pre-replicative period of 25 h; I_s attains values greater than 30% at 40–50 h, and then diminishes rapidly to 5% at 80 h. The I_m rises rapidly after 30 h to a maximum of 1·6% at 52 h; I_m then falls to 0·1% at 90 h. At 14 days after castration (Fig. 7b) there is a pre-replicative period of 20 h. The I_s attains values greater than 25% at 35–45 h and then diminishes to 5% at 90 h. The I_m rises rapidly 30 h after stimulation to a maximum of 1·8% at 48 h and then falls to 0·4% at 90 h.

The variation of I_s and I_m in the coagulating gland with time after the first

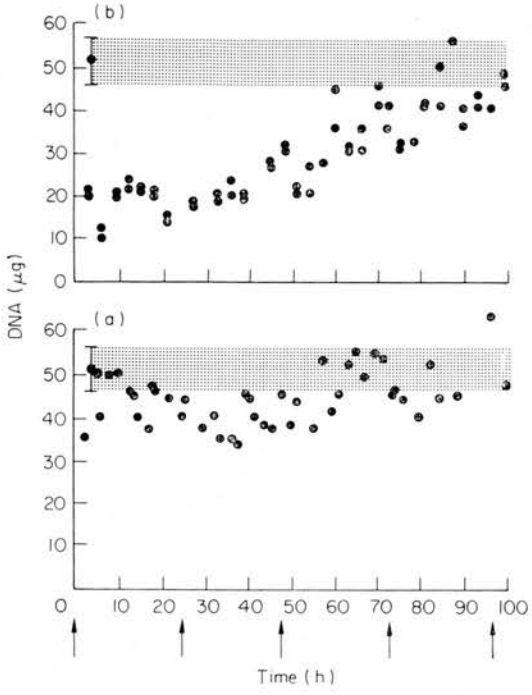

Fig. 6. DNA in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b). Shaded area represents mean $\pm$ s.d. in intact mice (n = 18).

injection of testosterone propionate is shown in Fig. 8. At 3 days after castration (Fig. 8a) there is a pre-replicative period of 40 h; I_s then rises to 25% at 55 h, and then falls to less than 5% at 70 h. The I_m rises after 45 h to a maximum of 1·4% at 66 h, and then falls to 0·1% at 80 h. At 14 days after castration (Fig. 8b) there is a pre-replicative period of 20 h. Both indices show distinct double peaks, with I_s reaching maxima at 30 h and 70 h and I_m approximately 40 h and 75 h.

The results of continuous labelling in the seminal vesicle is shown in Fig. 9. At 3 days after castration (Fig. 9a) the data are closely followed by the simulated line, assuming that 95% of the cells in the population enter DNA synthesis during the experiment. At 14 days after castration (Fig. 9b) the data are indicative of a

Table 1. Cell cycle parameters (h) in seminal vesicle and coagulating gland proliferation

	Seminal vesicle		Coagulating gland	
Days after castration	3	14	3	14
T_c		17·5		33·5†
t_{G1}		7·5		24·0
t_s	8·7★	8·0	8·1★	7·5
t_2	2·6★	2·0	2·8†	2·0

T_c = cell cycle time, t_{G1} = duration of the pre-synthetic period, t_s = duration of DNA synthesis, t_2 = duration of the post-synthetic period plus half the duration of mitosis.

★ Data analysed using the method of Gilbert (1972) for computer simulation of FLM curves.

† Value used in simulation by mathematical model of cell population growth (Alison *et al.*, 1974b).

Table 2. Tritium content in the seminal vesicle and coagulating gland after i.v. injection of [7α-^{3}H] testosterone

	Time after injection (min)	Days after castration 0	3	14
Seminal vesicle	5	128 ± 20 (4)	374 ± 94 (3)	296 ± 30 (4)
	30	152 ± 16 (4)	711 ± 70 (4)	375 ± 171 (8)
	60	172 ± 52 (4)	218 ± 45 (4)	272 ± 102 (8)
	120	132 ± 44 (4)	218 ± 56 (4)	191 ± 40 (6)
Coagulating gland	5	173 ± 9 (4)	339 ± 41 (4)	239 ± 25 (4)
	30	254 ± 35 (4)	501 ± 88 (4)	243 ± 54 (6)
	60	239 ± 23 (4)	165 ± 13 (4)	355 ± 232 (8)
	120	318 ± 114 (4)	154 ± 13 (4)	204 ± 20 (6)

The results are expressed as d.p.m. ± s.d. per μg DNA.
Values in parentheses = number of observations.

totally responsive population. The computer simulation achieved a poor fit to the experimental data.

The results of continuous labelling in the coagulating gland is shown in Fig. 10. At 3 days after castration (Fig. 10a) the data are closely followed by the simulated line assuming that 30% of the population enters DNA synthesis during the experiment. At 14 days after castration (Fig. 10b) the data are closely followed by the simulated line assuming that 85% of the cell population enters DNA synthesis during the experiment.

Table 1 shows the duration of cell cycle parameters measured by the FLM curves. A direct estimate of the cell cycle time was not possible in most experiments. The FLM method is not suited to the dynamic changes associated with a transitory proliferative response. Morley *et al.* (1973) however, showed that cell cycle parameters remained effectively constant throughout the duration of the proliferative response in the seminal vesicle of the 14 day castrate mouse. They concluded that the decline in labelling and mitotic indices was due to movement of cells out of the proliferative cycle during the later stages, and devised a model incorporating the concept of a decycling probability ($\vec{\eta}$); this parameter determines the proportion of newborn progeny that move out of the proliferative cycle. The mathematical model has predicted new experimental data when the parameters employed in the simulation were constrained by the FLM data (see Table 1 and Alison *et al.*, 1974b).

Table 2 shows the uptake and retention of tritium for up to 2 h after the injection of [7α-^{3}H]testosterone. In the intact animal the coagulating gland exhibited a higher uptake of radioactivity compared to the seminal vesicle. At 3 days after castration both tissues showed an increased uptake of radioactivity 5 min and 30 min after injection. The concentration of radioactivity was significantly higher ($P < 0{\cdot}01$) in the seminal vesicle compared to the coagulating gland both at 30 min and at 120 min after injection. At 14 days after castration the concentration of radioactivity in both tissues over the 2 h period of study was variable, though the different tissues exhibited a similar capacity.

Table 3 shows the analysis of the radioactivity by thin layer chromatography. In all the animals a large bulk of the radioactivity was associated with highly polar steroids which were not further identified. In the intact animals the major non-polar transformation product of testosterone was dihydrotestosterone in both accessory reproductive tissues. In the 3 and 14 day castrates the proportion of radioactivity associated with polar material increased from 0·5 h to 2 h. In the seminal vesicle of the 3 day castrates the proportion of the radioactivity associated with dihydrotestosterone was the same 30 min and 2 h after administration. In the coagulating gland however, this proportion fell markedly from 30 min to 2 h after injection. In the seminal vesicle of the 14 day castrates initially a higher proportion

of the radioactivity was associated with dihydrotestosterone compared to the coagulating gland. At 2 h after injection, however, the two tissues had an equal proportion of the radioactivity associated with the dihydrotestosterone fraction.

Autoradiograms were prepared from the tissues of mice castrated 3 days before which had been injected with 200 μCi [1,2,6,7(n)-^{3}H]testosterone (Fig. 11).

Table 3. Analysis of acetone-ethanol-extractable radioactivity from seminal vesicle and coagulating gland tissue by thin layer chromatography following i.v. injection of [7α-^{3}H] testosterone

Days after castration		0	3		14	
		Time after injection of testosterone (h)				
	Fraction	0·5	0·5	2·0	0·5	2·0
Seminal vesicle	A	74·80	64·19	83·18	74·18	93·54
	B	0·28	1·51	0·39	0·91	0·19
	C	0·24	1·76	0·30	2·93	0·10
	D	0·73	16·79	1·16	7·00	0·27
	E	21·61	14·50	14·71	14·30	5·76
	F	2·33	1·25	0·26	0·67	0·14
Coagulating gland	A	81·39	66·11	93·47	83·56	93·09
	B	0·21	0·93	0·24	0·79	0·18
	C	0·19	3·56	0·20	2·23	0·09
	D	0·79	15·61	1·12	9·45	0·50
	E	14·68	12·06	4·80	3·37	5·99
	F	2·74	1·73	0·17	0·60	0·15

Fraction A = highly polar material; fraction B = 5α-androstane-3α,17β diol; fraction C = 5α-androstane-3β,17β diol; fraction D = testosterone; fraction E = 5α-dihydrotestosterone; fraction F = androstene-3,17-dione and 5α-androstane-3,17-dione.

Values expressed as percentage of total radioactivity.

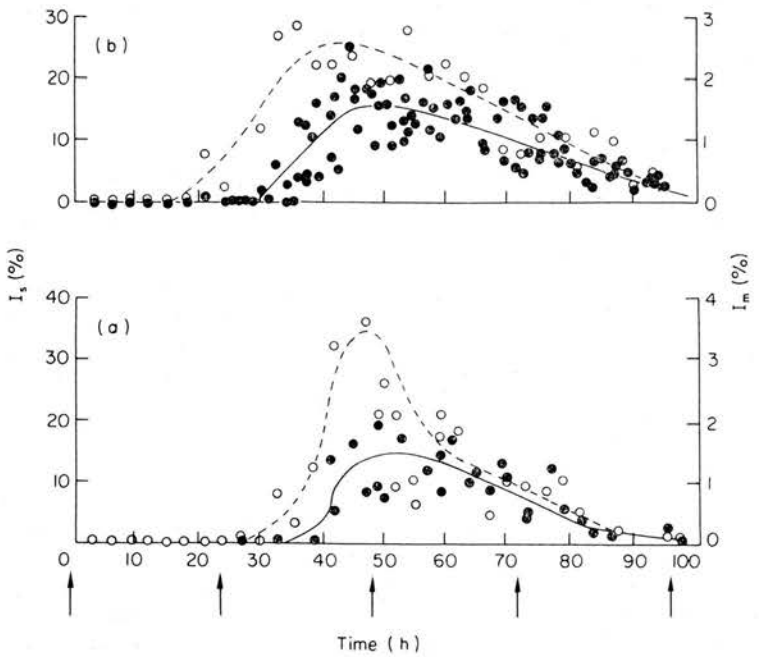

Fig. 7. I_s (○) and I_m (●) in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b).

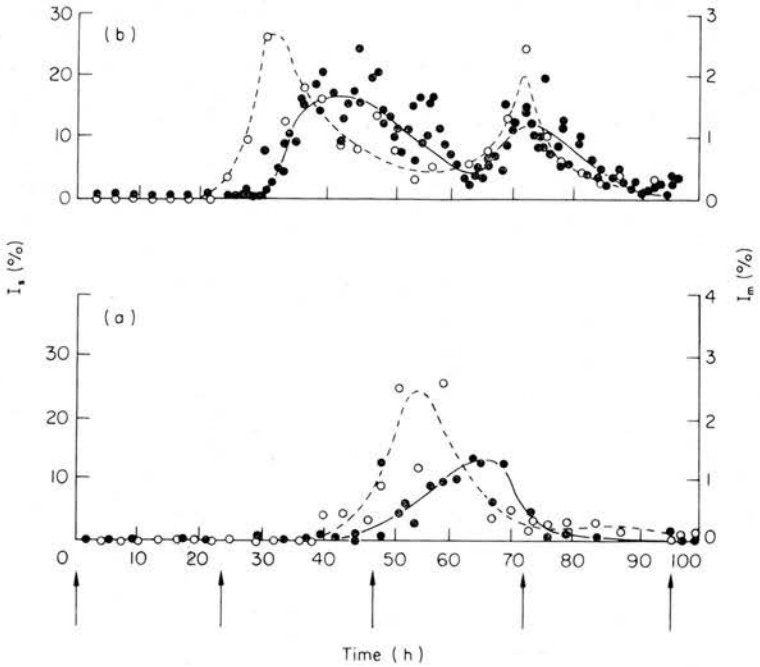

Fig. 8. I_s (○) and I_m (●) in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection administered 3 days after castration (a); the first injection administered 14 days after castration (b).

Animals were killed 5 min, 30 min and 120 min after administration of the steroids. The deposition of silver grains was heavier in the autoradiograms from the seminal vesicle (Fig. 11a, b, c). In the seminal vesicle the heaviest deposition was observed 5 min and 30 min after administration of steroid (Fig. 11a, b), with the majority of silver grains localized over nuclear areas. Silver deposition was considerably lighter over autoradiograms prepared from the coagulating gland (Fig. 11d, e, f). The heaviest deposition occurred 30 min after administration of steroid (Fig. 11). Subjective assessment indicated a non-specific localization of steroid in the coagulating gland (Fig. 11d, e, f).

DISCUSSION

The proliferative reaction occurring in the accessory reproductive organs in response to androgen is typical of induced DNA synthesis, comparable to the oestrogen-stimulated endometrium (Finn *et al.*, 1969), the liver after partial hepatectomy (Barbason *et al.*, 1975) and the isoproterenol-stimulated submandibular gland of the rat (Barka *et al.*, 1973). In all models of induced DNA synthesis the pre-replicative period observed before cells enter DNA synthesis suggests that before stimulation the sensitive cells are in a resting state, which has been called the Go state (Epifanova & Terskikh, 1969; Morley *et al.*, 1973).

An early manifestation of androgen action is a stimulation of RNA synthesis. Wicks & Kenny (1964), using radioactively labelled nucleotides, demonstrated a 50% rise in uptake of radioactivity into seminal vesicle RNA only 70 min after

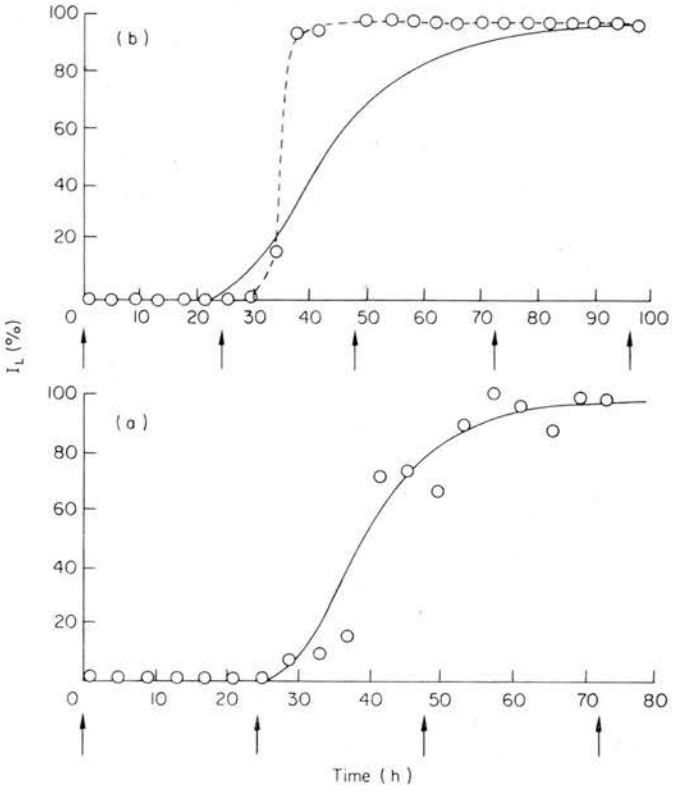

Fig. 9. Percentage labelled cells in the seminal vesicle of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection of testosterone administered 3 days after castration (a); the first injection of testosterone administered 14 days after castration (b). Tritiated thymidine was given every 4 h at a dose of 0·5 μCi/g body weight.

the i.p. injection of 10 mg testosterone. Williams-Ashman *et al.* (1964) demonstrated the ability of androgens to stimulate RNA polymerases, concerned particularly in the production of prostatic polyribonucleotides which function as templates in protein biosynthesis. The results of Sasaki & Baserga (1970) indicated gene activation commenced in mice salivary glands only several hours after the administration of isoproterenol. Isotalo and Santi (1975) observed that all the main species of RNA isolated from the ventral prostate of the castrated rat showed a marked increase in labelling 12 h after testosterone treatment.

The induction of DNA synthesis in androgen dependent tissue is preceded by a pre-replicative period of duration 15–30 h. Tuohimaa *et al.* (1973) observed a pre-replicative period of 20–30 h before the incorporation of thymidine into DNA in the seminal vesicle, coagulating and prostate of the rat castrated 1 month before; Lesser & Bruchovsky (1973) detected the incorporation of [Me-^{3}H]thymidine into the prostatic nuclear DNA 24–36 h after the first injection of dihydrotestosterone in 7 day castrate rats.

This study has shown that the apparently predictable temporal sequence of events making up the proliferative reaction is altered by challenging animals at different times after castration. The pre-replicative period in the seminal vesicle

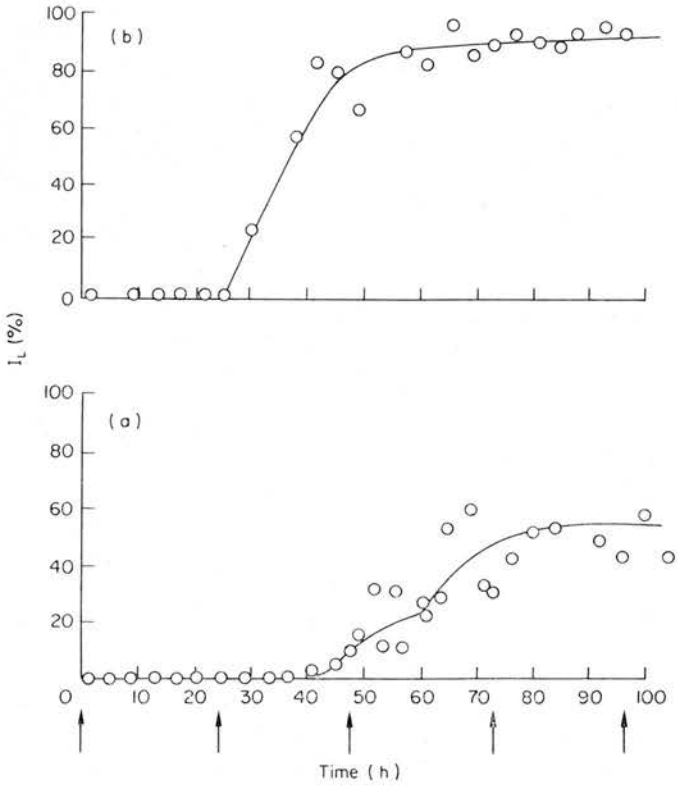

Fig. 10. Percentage labelled cells in the coagulating gland of the castrate male Balb/c mouse during daily treatment with 250 μg of testosterone propionate (arrows). The first injection of testosterone administered 3 days after castration (a); the first injection of testosterone administered 14 days after castration (b). Tritiated thymidine was given every 4 h at a dose of 0·5 μCi/g body weight.

of both groups of post-operative castrated mice was rather similar, 20–25 h. In contrast however, a pre-replicative period of 40 h was observed in the coagulating gland of the 3 day castrated mice; a quiescent phase which was double the duration of that observed during the proliferative reaction in the same tissue of the 14 day castrated mice. Concomitantly an increase in RNA, well established as an early biochemical manifestation of androgen action, is comparatively delayed during the response in the coagulating gland of the 3 day castrated mice.

The continuous labelling experiments established that the seminal vesicle was almost totally responsive at both times after castration, whilst the androgen sensitivity of the coagulating gland increased as time elapsed after castration. The magnitude of the response in both tissues would seem to be most likely related to the extent of cell loss. In the seminal vesicle of the 3 day castrated mouse, where the amount of DNA was half the normal complement, computer simulation of the [^{3}H]thymidine labelling and mitotic indices suggested $\vec{\eta}$ to be close to 1·0, i.e. the proliferating cells divided only once. At 14 days, where the amount of DNA was only 30% of the normal complement, simulation suggested $\vec{\eta}$ to be initially 0·75, i.e. 25% of newborn progeny from the division of the androgen sensitive cohort, proceeded through at least one more division cycle. In the coagulating gland of 3 day

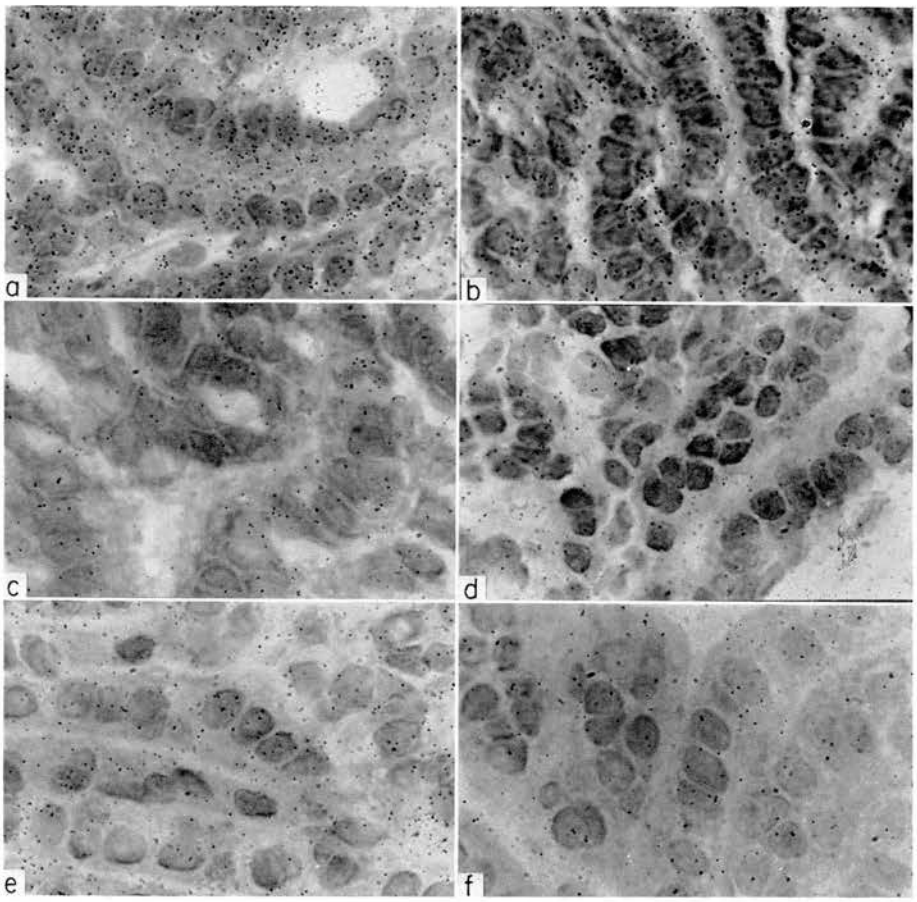

Fig. 11. Autoradiographs of 6 μm thick sections of the mouse seminal vesicle and coagulating gland after i.v. administration of 200 μCi [1,2,6,7(n)-^{3}H] testosterone Mice were castrated 3 days prior to administration. Values in parentheses are the times at which animals were killed after administration of the steroid. (a) Seminal vesicle (5 min, $\times$1000); (b) seminal vesicle (30 min, $\times$1000); (c) seminal vesicle (120 min, $\times$1000); (d) coagulating gland (5 min, $\times$1000); (e) coagulating gland (30 min, $\times$1000); (f) coagulating gland (120 min, $\times$1000).

castrated mice, the amount of DNA was still at least 80% of normal, hence the restoration of the normal complement required only a small transitory response involving only 30% of the cell population. At 14 days, when the amount of DNA was half the normal complement, a prominent biphasic response followed testosterone administration which entailed 85% of the cell population entering the proliferative cycle. This increased androgen sensitivity in the coagulating gland from day 3 to day 14 after castration represented an absolute increase in the number of responsive cells, and could not be accounted for by a preferential loss of non-responsive cells from the population.

The observation that the pre-replicative phase in the coagulating gland was

dependent upon the time elapsed since castration was very surprising, particularly as this phase became shorted the longer the time left in an unstimulated state after castration. Bucher *et al*, (1964) studying regenerating rat liver observed that as animals grew older, there was an increasingly longer lag in the initiation DNA synthesis in the liver following partial hepatectomy.

Studies on the uptake, metabolism and localization of tritiated testosterone were undertaken to investigate a possible cause–effect relationship between hormone metabolism and hormone action. Three days after castration, at which time the seminal vesicle and coagulating gland exhibit the largest difference in response, the maximum uptake and subsequent retention of administered radioactive testosterone were consistently greater in the seminal vesicle, the potentially more proliferative tissue. At 14 days after castration when the tissues exhibit a similar response to testosterone propionate, the pattern of uptake and retention of labelled testosterone was largely similar. The increased uptake of radioactivity soon after castration, and a subsequent reduction in capacity later are comparable with the observations of Buric *et al.* (1972). The pronounced increase in uptake 3 days after castration can be explained by a reduction in endogenous hormone, freeing receptor protein for exogenously administered radioactive testosterone. The reduced capacity of both tissues at 14 days indicates a reduction in available receptor protein.

Although final identification of labelled steroid metabolites was not performed, the results (Table 3) indicated a rapid conversion of labelled testosterone in the target tissues. In all experiments a high proportion of the radioactivity was associated with highly polar steroids; they may represent conjugates, formed in the target tissues themselves or in peripheral sites such as the liver and blood. At 3 days after castration it may be of considerable significance that 2 h after the administration of labelled testosterone, the coagulating gland retains very little radioactivity associated with dihydrotestosterone in comparison to the seminal vesicle. The inability at this time to conserve dihydrotestosterone, the metabolite assumed to play a central role in stimulating RNA transcription, may be in part responsible for the extraordinarily long pre-replicative phase observed in the response of the coagulating gland to exogenously administered testosterone propionate. Dry mount autoradiography was carried out to investigate the localization of steroid in the proliferatively different target tissues of the 3 day castrated mouse. A higher deposition of silver grains was observed in autoradiograms of the seminal vesicle (Fig. 11a, b, c) compared to the coagulating gland (Fig. 11d, e, f). This was in accordance with our measurements of the uptake of administered [7α-^{3}H] testosterone by scintillation counting (Table 2). Autoradiographs of the seminal vesicle 5 min, 30 min and 120 min (Fig. 11a, b, c) after the administration of radioactive testosterone revealed that the silver grains were primarily located over nuclear areas, no such nuclear concentration was observed in the autoradiograms of the coagulating gland. These results indicated more nuclear binding of steroid in the seminal vesicle of the 3 day castrated mouse in comparison to the coagulating gland. Studies of testosterone metabolism (Table 3) indicated the greater ability of the seminal vesicle to retain dihydrotestosterone, the only metabolite capable of being nuclear bound. The apparent lack of nuclear binding in the coagulating gland (Fig. 11d,e, f) may play a significant role in lengthening the duration of the pre-replicative phase during the response in the 3 day castrated mouse. Nuclear binding necessitates dihydrotestosterone and testosterone permeating the nuclear membrane; such a membrane may present a physical barrier to this vital step in the initial stages of the response in the coagulating gland of the 3 day castrated mouse. This barrier may be conquered at a later stage of the response by a change in the composition of the nuclear membrane; indeed Pollard & Martin (1975) have shown that nuclear

non-histone proteins are susceptible to oestrogens during the response in the uterus of the castrate mouse.

ACKNOWLEDGMENTS

We are grateful to Mrs Elizabeth Wallace, Mrs Sandra Gillot and Miss Barbara James for skilled technical and graphical assistance. We are grateful to Miss Elizabeth Wark for typing the manuscript. We gratefully acknowledge the financial support of the North of England Branch of the Cancer Campaign.

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J. Endocr. (1975), **65**, 353–361 *Printed in Great Britain*

THE PROLIFERATIVE RESPONSE OF THE COAGULATING GLAND OF THE CASTRATED MOUSE UNDER CONTINUOUS ANDROGEN STIMULATION: AN EXPERIMENTAL AND COMPUTER SIMULATION MODEL

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(*Received* 22 *July* 1974)

SUMMARY

The proliferative response of the coagulating gland of the castrated male mouse has been examined during continuous treatment with testosterone propionate. Fourteen days after castration, s.c. daily injections of testosterone propionate were begun. Mitotic (I_m) and labelling (I_L) index values were obtained at 3 h intervals for up to 100 h after the initial injection. These showed a biphasic response, in which I_L reached a maximum at 30 and 70 h, and I_m at approximately 45 and 75 h. Fraction-labelled mitoses (FLM) curves were begun 24, 48, and 72 h after the first androgen injection. In each curve the first wave of labelled mitoses rose to 100 % and showed a square form indicating little spread in the durations of the G_2 and S phases. Values of 7·5, 1·3 and 0·7 h, were obtained for the durations of DNA synthesis (t_s), the post-synthetic period (t_{G2}) and of mitosis (t_m) respectively. In none of the FLM curves was it possible to demonstrate a second wave of labelled mitoses and direct measurement of the cell cycle time (T_c) was not obtained. Continuous tritiated thymidine labelling indices revealed that after a latent period of 25 h, DNA synthesis began and labelling rose rapidly to 80 % by 45 h and then more slowly to 95 % by 97 h. Cell population changes during androgen stimulation estimated from measurements of total glandular DNA indicated that the number of cells present in the glands remained constant during the first 30 h after stimulation and thereafter increased to approximately 2·3 times the original value.

The data are compared with a mathematical model which assumes that the cell population of castrated mice when stimulated passes from a G_0 compartment through successive waves of DNA synthesis and mitosis. After each cell division the cells may leave or remain in the proliferative cycle. This model has been subjected to computer simulation using the cell cycle parameters obtained in the kinetic experiments. There was good agreement between the simulation and experimental results in the I_m and I_L curves, continuous labelling, and total cell number experiments. The simulation of FLM curves was less successful. Although the first wave of labelled mitoses was clearly seen the model predicts a distinct second wave of labelled mitoses. It is concluded that this does not appear because of variation in the duration of G_1.

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INTRODUCTION

The coagulating glands of the rodent lie parallel and medial to the larger seminal vesicles and open by paired ducts into the urethra at the base of the bladder. They were first recognized as separate entities by Walker (1910) who described the coagulative properties of the secretion on mixing with the fluid of the seminal vesicle. Like the other glands of the prostate complex, the coagulating gland is mainly dependent on testicular androgens for maintenance in the functional state and castration causes atrophy and a diminution in mitotic activity (Allen, 1958). There is also a fall in total glandular DNA, RNA and protein to a similar degree to that found in the seminal vesicle and ventral prostate (Kochakian, 1963). After treatment with androgens, the coagulating gland shows the characteristic sequence of proliferative changes seen in models of induced DNA synthesis [see review by Cooper (1971) and the elegant study of Barka, Chang & van der Noen (1973)]. After stimulation there is a latent period of some 15–20 h, then a wave of DNA synthesis followed by mitotic activity. Under continued hormonal treatment there is evidence of functional activity, cellular hypertrophy, and increased secretion, associated with a fall in DNA synthesis and mitotic activity.

Structurally the coagulating gland is similar to the seminal vesicle but histological and ultrastructural differences are apparent. The proliferative characteristics of the rodent coagulating gland have received little study. In the castrated mouse, Allen (1956, 1958) showed that after a single dose of testosterone propionate or oestradiol there was a vigorous mitotic response after the characteristic lag period of some 20 h. Mitotic indices after several hours of colchicine treatment gave little precise information about cell kinetic parameters. More recently Tuohimaa, Oksanen & Niemi (1973) have examined the uptake of tritiated thymidine in seminal vesicles, coagulating glands and ventral prostates of castrated rats treated daily with testosterone or dihydrotestosterone. Although labelling indices suggested considerable variability in response, the data were insufficient to allow calculation of cell cycle parameters.

In previous publications (Morley, Wright & Appleton, 1973; Appleton, Morley & Wright, 1973) we described the cell cycle parameters of the seminal vesicle of the castrated mouse during testosterone stimulation, and showed that the data were consistent with a model composed of a G_0 compartment from which cells passed after hormonal stimulation into DNA synthesis and mitosis. The probability of daughter cells remaining in the proliferative cycle was expressed as $\bar{\eta}$, and the probability of their entering a non-proliferative, differentiated compartment was termed $\bar{\eta}$ following the notation of Bresciani (1968) (see Fig. 1). Computer simulation showed that this model appeared to be consistent with the cell cycle parameters obtained for the androgen-stimulated seminal vesicle of the castrated animal. We now present experimental data from the androgen-stimulated coagulating gland. Computer simulation using the above model demonstrated that the proliferative changes may also be described in these terms.

The abbreviations used are as follows: T_c, duration of cell cycle; t_s, duration of DNA synthesis; t_{G1}, duration of the pre-synthetic period; t_{G2}, duration of the post-synthetic period; t_m, duration of mitosis; I_m %, mitotic index per cent; I_L %, labelling index per cent.

METHODS

The experimental procedures were as described by Morley *et al.* (1973). Male Balb/c mice aged 3 months were castrated. Fourteen days later daily s.c. injections of testosterone propionate (250 μg) dissolved in sesame oil were begun and continued daily until the animal was killed.

To obtain I_m and I_L values throughout the period of stimulated proliferation, animals were killed by cervical dislocation at intervals of 3 h for up to 100 h after the initial injection of androgen. Tritiated thymidine (sp. act. 5·0 Ci/mM, Radiochemical Centre, Amersham) was injected i.p. at a dosage of 1 μCi/g body weight 1 h before death. At autopsy coagulating glands were fixed in Carnoy's solution. Multiple sections were cut at a thickness of 5 μm. Autoradiographs were prepared using Kodak A.R. 10 stripping film and developed in Kodak D 19b. Sections were exposed for 2 weeks and stained with Harris's haematoxylin. All cells with more than 5 grains per nucleus were counted as labelled.

The cell cycle parameters were measured by the fraction labelled mitoses (FLM) method, starting at 24, 48 and 72 h after the first injection of testosterone propionate. At these times tritiated thymidine was given by the route and dosage described above. To obtain FLM curves 100 mitotic cells in all stages of division were counted for each point. The durations of cell cycle parameters were measured from the FLM curves at the 50 % level (Quastler & Sherman, 1959). Continuous labelling data were obtained by the injection of 0·5 μCi [^{3}H]thymidine/g immediately after the initial injection of androgen and thereafter at 4 h intervals until 96 h. An animal was killed 1 h after each injection of tritiated thymidine.

Measurement of cell population size was performed by determining the DNA content of coagulating glands removed at 3 h intervals for up to 100 h. The glands were rapidly frozen in liquid nitrogen. Total DNA content was measured by a modification of the method of

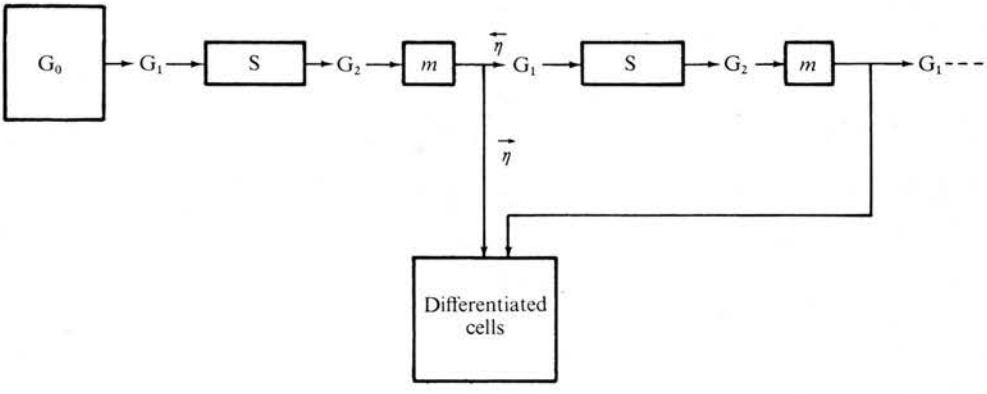

Fig. 1. A simple model of the stimulated cell kinetic response in the coagulating gland and seminal vesicle following daily treatment with testosterone propionate, on which computer simulation is based. See text, page 354, for definition of abbreviations.

Schneider (1946). Homogenates (10 %, w/v) were prepared at 4 °C in 0·1 M-Tris–EDTA, pH 8·0. The nucleic acids, together with protein, were precipitated by the addition of ice-cold 10 % trichloroacetic acid. The acid-insoluble material was heated for 30 min with 2 % perchloric acid at 90 °C. DNA was measured in the cooled supernatant by the modified diphenylamine reaction described by Burton (1968).

Cells in DNA synthesis were assumed to contain 3N amounts of DNA. Since I_L values were available at all periods throughout the experiment it was possible to obtain a corrected value for the number of diploid cells from measured DNA. Similarly cells in G_2 and mitosis with a ploidy of four would lead to an overestimation of cells in these phases. The size of the tetraploid cohort was estimated from the phase durations as 27 % as large as the cohort in DNA synthesis. Both these corrections are approximations and assume cells enter and proceed through DNA synthesis asynchronously giving a mean ploidy of 3N for the

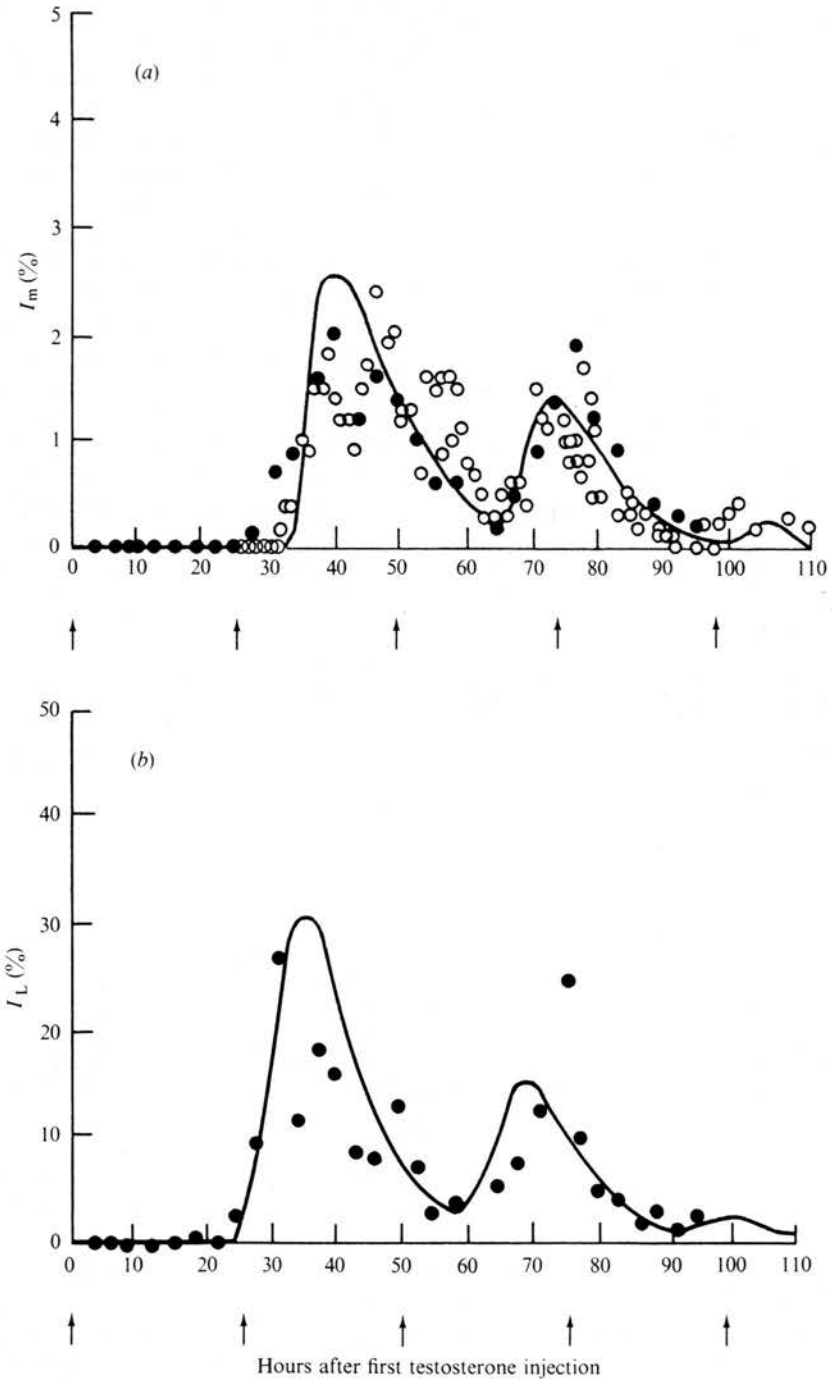

Fig. 2 (*a*) Mitotic index curve (solid circles) in the coagulating gland of the castrated male Balb/c mouse during daily treatment with 250 μg testosterone propionate (arrows). Open circles are results from animals used in the fraction-labelled mitoses experiments. The continuous line shows the computer simulation. I_m, mitotic index. (*b*) Labelling index curve (solid circles) in the coagulating gland of the castrated male Balb/c mouse during daily treatment with 250 μg testosterone propionate (arrows). The continuous line shows the computer simulation. I_L, labelling index.

thymidine-labelled population. From these adjusted measurements the number of nuclei was calculated assuming 6·2 pg DNA/nucleus (Enesco & Leblond, 1962).

The system described in Fig. 1 was simulated by a mathematical model incorporating the following features. (i) All cells are initially in a G_0 phase. (ii) A proportion of the cells leave G_0 and pass through G_1, S, G_2 and *m* phases. (iii) The rate at which these cells leave G_0 is a function of time only. (iv) All cells take the same time to pass through a given phase. (v) Progeny may leave the cycle or proceed through a further cycle. (vi) The decycling probability at the end of each cycle may be constant, or may vary with the total number of cells in the system, or the number of cells that have left the system, or by any other function of time. It may not exceed unity. (vii) Labelling of cells in the S phase is assumed to be instantaneous. If this is not so estimates of duration of S (t_s) must be decreased and estimates of duration of G_1 (t_{G1}) increased by the duration of action of the labelling agent.

The mathematics of this model and its ability to simulate the cell kinetic data obtained from androgen stimulation of the castrated mouse seminal vesicle have been described fully in a previous publication (Appleton *et al.* 1973).

RESULTS

Fig. 2(*a*, *b*) shows the mitotic and labelling indices following androgen-stimulation of the coagulating gland. The closed circles indicate mitotic indices from animals used to obtain the labelling indices. The remaining points were obtained from animals used in FLM experiments. Both sets of data show distinct double peaks, with I_L reaching a maximum at 30 and 70 h and I_m at approximately 45 and 75 h. The latent period of some 20 h between the first testosterone propionate injection and the onset of DNA synthesis (Fig. 2*a*) is characteristic of induced DNA synthesis. If the two peaks are tentatively assumed to indicate division of a partially synchronized population then the time elapsing between peaks gives an approximate measure of the cycle time T_c = 40 h.

Fig. 3(*a*, *b*, *c*) shows FLM curves begun 24, 48 and 72 h after the first testosterone propionate injection. In each experiment, the first wave of labelled mitoses rose to 100 % and took a square form indicating relatively little spread in the durations of the G_2 and S phases. It should be noted that unlike the seminal vesicle there is no distinct second wave of labelled mitoses, so that a direct measurement of T_c is not obtainable. The duration, in hours, of cell cycle parameters used in simulation of coagulating gland proliferation were as follows: T_c, 33·5; t_{G1}, 24; t_s, 7·5; t_{G2}, 1·3; t_m, 0·7 [85 % of cells leave G_0 compartment, Erlangian parameter 2, $\bar{\eta}$ = 0·5+0·003*n* (where *n* = number of cells in differentiated compartment) until $\bar{\eta}$ = 1 and thereafter remains constant] (computer simulation, solid line, Fig. 2*a*, *b*).

The ability of the computer simulation to fit the FLM curves is less satisfactory (Fig. 3, solid lines), since there is no indication of a second peak of labelled mitoses. This suggests that assumption (iv) in the mathematical model is not fulfilled, and since it is evident from the FLM curves that there is little variation in the durations of S, G_2 and *m*, it is probable that G_1 shows considerable variation.

The results of continuous labelling are given in Fig. 4. There is a latent period of 25 h before the onset of DNA synthesis followed by a rapid rise to 80 % by 45 h then a slower increase to reach 95 % at 97 h. The continuous labelling data are closely simulated by the mathematical model if it is assumed that only 85 % of the cells in the G_0 compartment leave during the experiment. This value was obtained after multiple simulations in which only the percentage of cells leaving G_0 was altered.

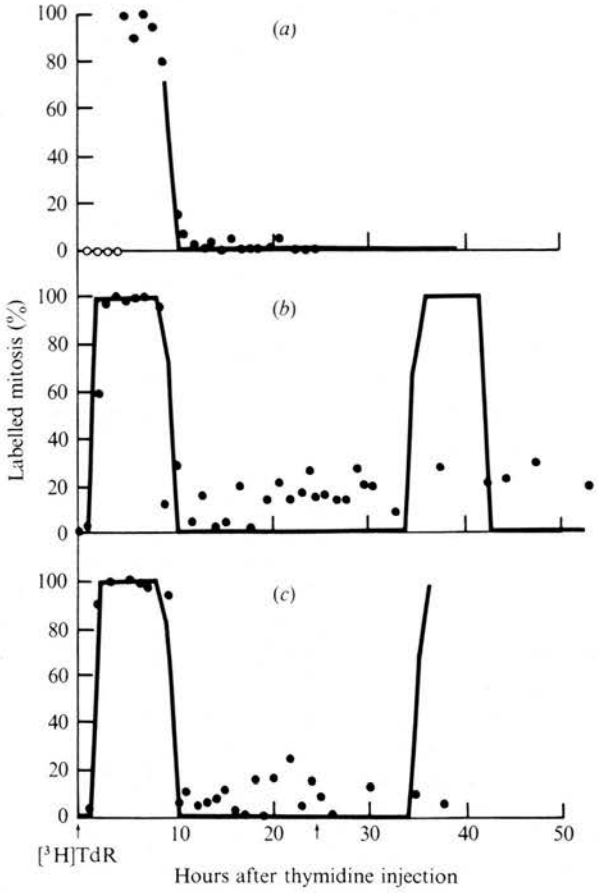

Fig. 3. Fraction-labelled mitosis curves in the coagulating gland of the castrated male Balb/c mouse during daily treatment with 250 μg testosterone propionate (arrows). Tritiated thymidine was given (*a*) 24, (*b*) 48 and (*c*) 72 h after the initial androgen injection. The thick line shows computer simulation. Open circles (*a*) are points at which no mitotic figures were observed.

Cell population changes following androgen stimulation

During the first 25 h of the experiment there was no alteration in cell number. The mean cell number in this period was taken as 100 % and all subsequent estimates of cell number were related to this value. Fig. 5 shows the correspondence between the simulation and the experimental estimates of cell number. There is too much variation in the experimental data to distinguish the shallow fluctuations in the simulated curve caused by successive waves of cell division.

DISCUSSION

Computer simulation models are now a familiar feature of cell kinetic studies. Most have been concerned with analysis of FLM data. Many incorporate optimizing methods to find the cell cycle parameters which best fit given FLM data. Indeed many biological data are not sufficiently precise to warrant the sophistication of the methods used to analyse them. Most simulation models have considered systems in steady state, or exponential growth conditions. Models attempting to describe the kinetic behaviour of induced DNA synthesis and cell proliferation are less common. An early example was that of Iversen & Bjerknes (1963)

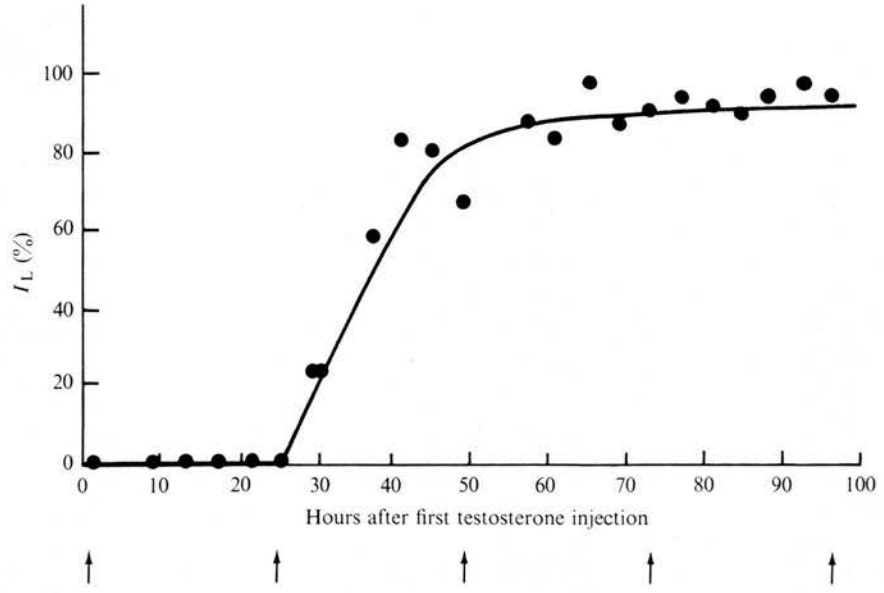

Fig. 4. Percentage of labelled cells in the coagulating gland of castrated male Balb/c mouse during daily treatment with testosterone propionate (arrows) and 4-hourly injections of tritiated thymidine (0·5 μCi/g). I_L, labelling index.

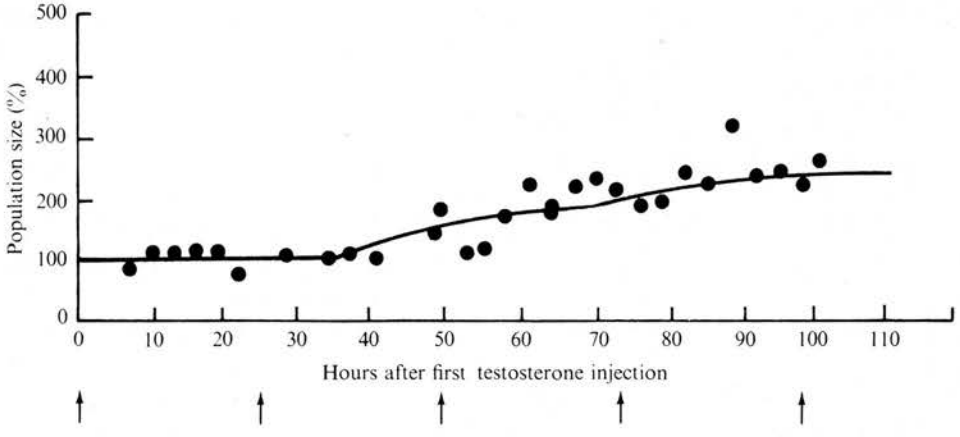

Fig. 5. Cell population increase in coagulating gland of castrated male Balb/c mouse during daily treatment with testosterone propionate (arrows) as determined from total gland DNA content, and with 100 % value being the mean observations up to 25 h.

describing the proliferation of mouse epidermis following treatment with 3-methylcholanthrene in benzene. Johnson (1969) has shown that cell proliferation in the rat liver remaining after partial hepatectomy can be predicted from a mathematical model in which increased cell size is the trigger of DNA synthesis.

The model described here was developed as a hypothesis of the cell kinetic events following androgen stimulation in the castrated mouse seminal vesicle. With I_m and I_L data, and FLM curves obtained at intervals throughout the proliferative response, a model was described in which the cardinal feature was the loss of cells from the proliferative cycle following cell

division. The probability of this event was termed the decycling probability ($\bar{\eta}$). It has been shown that the biphasic I_m and I_L curves obtained in the coagulating gland can be simulated when the model is limited by the known cell cycle parameters determined during the proliferative response (see Figs 1 and 2). The similarities between the FLM curves at 48 and 72 h and the square form of the curves indicate little variation in t_s, t_{G2} and t_m. Since there is no second peak, the FLM data suggest that there may be sufficient variation in t_{G1} to invalidate feature (iv) of the mathematical model, i.e. that all cells take the same time to pass through a given phase.

It can be suggested that a biphasic proliferative response is the result of proliferation of two cell populations with different cell proliferation parameters. Rowlatt & Franks (1964) have described a population of reserve cells or intercalated cells in the mouse seminal vesicle. However, inspection of the continuous labelling curve (Fig. 5) shows that at 60 h almost all the cells destined to leave G_0 have done so. This observation suggests that any secondary population is too small to account for the second wave of labelled cells.

It is paradoxical that in a proliferative response which is biphasic, a second peak is not obtained in the FLM analysis (Fig. 3*b*). This finding may suggest that the division of a cell early or late in the first proliferative wave is not correlated with the duration of T_c in its progeny. The 24 h duration of the lag phase between testosterone propionate administration and cell division appears remarkably constant. Lajtha (1963) has suggested that this is indicative of a population of cells in a G_0 phase. Schmid, Schell & Heyder (1973) in a study on cell proliferation in the seminal vesicles of castrated rats have shown that the initial lag phase appears to be independent of the stimulating dose of androgen, and of the magnitude of the subsequent cell proliferative response.

There is good agreement between the computer prediction of increase in cell number, and population size as measured by total DNA content. More accurate measurement of mean nuclear DNA content in the mouse prostate and a determination of the number of tetraploid cells during the proliferative response are required. Within the limits of the methods employed, this agreement suggests that the computer simulation and the model on which it is based represent a reasonable hypothesis of the kinetic behaviour of the androgen-stimulated cells of the coagulating gland of the castrated mouse.

The authors would like to thank Professor A. G. Heppleston and Professor D. J. Newell for their continued interest and support. Mrs M. Moody, Mr H. Elliot and Miss S. James provided excellent and unstinting technical and graphic assistance. We are indebted to the North of England branch of the Cancer Campaign for generous financial assistance.

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J. Endocr. (1974), **62**, 527–536
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EFFECT OF DEXAMETHASONE ON CELL POPULATION KINETICS IN THE ADRENAL CORTEX OF THE PREPUBERTAL MALE RAT

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(*Received* 6 *December* 1973)

SUMMARY

The effect of a single injection of dexamethasone on adrenocortical cell proliferation was studied in prepubertal male rats using tritiated thymidine. After a short latent period, all zones of the adrenal cortex showed a rapid decrease in both labelling and mitotic indices. After a prolonged period when very low indices were apparent, there was a rapid rise in both proliferative indices with most zones showing a considerable increase above control values.

A more detailed study of the initial depression showed that after a latent period of about 5 h the labelling index fell approximately 8 h before the mitotic index. This differential response in the labelling and mitotic indices was consistent with a block in the cell cycle late in the pre-DNA synthetic interval of the cell cycle (G_1), with cells being prevented from entering DNA synthesis. This hypothesis was also supported by an experiment involving continuous labelling of control and dexamethasone-treated animals; again after a latent period of 5–6 h, the rate of increase of the continuous labelling index fell as cells became blocked in late G_1.

By analogy with other tissues, results are interpreted in terms of a direct action of dexamethasone on adrenocortical cells; this steroid-sensitive step in the cell cycle may be important in the control of growth in the adrenal cortex.

INTRODUCTION

The atrophy induced in the inner zones of the adrenal cortex by hypophysectomy has been appreciated for many years and has led to proposals that adrenocorticotrophic hormone (ACTH), in addition to its controlling role in steroid production, has an important action in maintaining the structural integrity of the adrenal cortex (Garren, Gill, Masui & Walton, 1971). Despite recent rapid progress in the understanding of steroidogenesis and its hypothalamic control, the action of ACTH on adrenal cell populations has been largely neglected.

On the other hand, several workers have studied the action of ACTH on adrenal nucleic acids, in particular DNA (Farese & Reddy, 1963; Bransome, 1968). Masui & Garren (1970) have shown that after a latent period, administration of ACTH increases adrenal DNA content and, therefore, presumably adrenocortical cell

number. In addition, Masui & Garren (1970) have demonstrated that ACTH increases rates of incorporation of tritiated thymidine ([^{3}H]Tdr) into DNA, and also stimulates adrenal DNA polymerase and thymidine kinase activity.

These indirect observations indicate that ACTH does play a role in the control of adrenocortical cell replication. The manner in which this control is exerted is at present obscure; any unified theory of action would have to incorporate the fact that adrenocortical cytogenesis, at least in the prepubertal animal, is largely a function of the outer cortical zones, in particular the z. glomerulosa (Wright, Voncina & Morley, 1973).

Direct observation of ACTH-induced cell proliferation in the rodent adrenal cortex using [^{3}H]Tdr has proved rather disappointing, with very variable responses both in the timing and magnitude of the response (Reiter & Hoffman, 1967). Consequently we have approached the problem of growth control in adrenocortical cells in a rather different way by the administration of dexamethasone to intact prepubertal male rats. A preliminary report of this work has already appeared (Wright, 1971*a*), but here we describe the action of dexamethasone on the various anatomical zones of the adrenal cortex and relate the findings to adrenocortical cytogenesis.

MATERIALS AND METHODS

Male Wistar rats aged 14 days and weighing 19–25 g were given a single intraperitoneal injection of dexamethasone phosphate (Merck, Sharp and Dohme) in a dosage of 3 μg/g body weight. Control animals received a similar volume of 0·9 % saline. In an attempt to minimize the effect of diurnal variation these injection procedures were carried out at 09.00 h.

Tritiated thymidine (Radiochemical Centre, Amersham, Bucks.), of specific activity 5 Ci/mmol, was administered by intraperitoneal injection in a dosage of 1 μCi/g body weight. The concentration of [^{3}H]Tdr used was 100 μCi/ml. The animals were returned to their mothers after each injection procedure.

Animals were killed by cervical dislocation. The adrenals were fixed for 5 h in Carnoy's fluid. Serial transverse sections were cut at a thickness of 3 μm, and autoradiographs were prepared as previously described (Wright, 1971*b*), employing an exposure period of 28 days. Only sections clearly showing the relationships between the cortical zones were studied. Following the procedure of Reiter & Pizzarello (1966), the cortex was divided for analytical purposes into z. glomerulosa, transition zone, outer z. fasciculata, inner z. fasciculata and z. reticularis. Differentiation into recognizable zones is well established by 14 days (Ford & Young, 1963; Wright *et al.* 1973), and the criteria for the recognition of the various zones were those of Ford & Young (1963). In each area the labelling index (LI) was determined by scoring 2000 nuclei, and the mitotic index (MI) by scoring 4000 nuclei. The labelling index is here defined as the percentage of labelled cells, and the mitotic index as the percentage of cells in mitosis, in the population studied. Hunt & Hunt (1966) and N. A. Wright (unpublished observations) have shown that no statistically significant differences are apparent when these proliferative indices are compared for each zone in the right and left adrenal of the same animal. Consequently in an individual animal the results for each zone of the right and left adrenals were combined and the mean taken as the result for that animal.

The criteria for regarding a cell as labelled were those of Wright (1971*c*). In the determination of the mitotic index all phases of mitosis were included and the criteria for the definition of mitotic phases were those of Clarke (1970). All slides were randomized before counting commenced.

Experiment 1

In a pilot experiment three animals were killed every 6 h for 96 h after injection of dexamethasone. Control animals were killed in groups of three every 6 h for 30 h, and then at 48, 72 and 96 h after injection of 0·9 % saline. One hour before death all animals received [^{3}H]Tdr.

Experiment 2

The action of dexamethasone was followed more intensively by killing animals in pairs every hour for 30 h after dexamethasone treatment. In this experiment animals again received [^{3}H]Tdr 1 h before death.

Experiment 3

The period immediately after administration of dexamethasone was next studied by a continuous labelling experiment. Dexamethasone-treated experimental and saline-treated control animals were given hourly injections of [^{3}H]Tdr in a dosage of 0·5 μCi/g body weight. The lower dose was chosen in order to limit the amount of ^{3}H given to each animal. One animal was killed every hour and the LI obtained for each zone as previously described.

RESULTS AND INTERPRETATION

Experiment 1

The mean values for the labelling and mitotic indices measured at 6-h intervals after dexamethasone injection are shown for the z. glomerulosa and outer z. fasciculata in Fig. 1. In the z. glomerulosa the proliferative indices both remained approximately constant for the first 6 h before a sharp fall to reach very low values by 24 h after dexamethasone treatment. These low values were maintained until about 48 h, when a steady increase ensued, and this zone showed a prominent increase above initial, normal values; indeed the indices had not returned to normal levels by 96 h after dexamethasone treatment, the end of this experiment. The outer z. fasciculata shows a similar response (apart from lower initial values for the labelling and mitotic indices in the outer z. fasciculata). After the same latent period of about 6 h a rapid fall in both indices occurred, with very low values again apparent at 24 h. However, the depression lasted until 72 h after dexamethasone treatment, when a very sharp rise occurred. The results in the remaining zones studied were effectively the same.

Measurements of proliferative indices in the saline-treated control animals showed no decrease in any zone. Figure 2 shows the results obtained for the outer z. fasciculata. It is apparent that no depression of either proliferative index occurred over the experimental period

Experiment 2

In this experiment animals were killed at hourly intervals after dexamethasone administration and the results are given in Fig. 3. In the z. glomerulosa the labelling

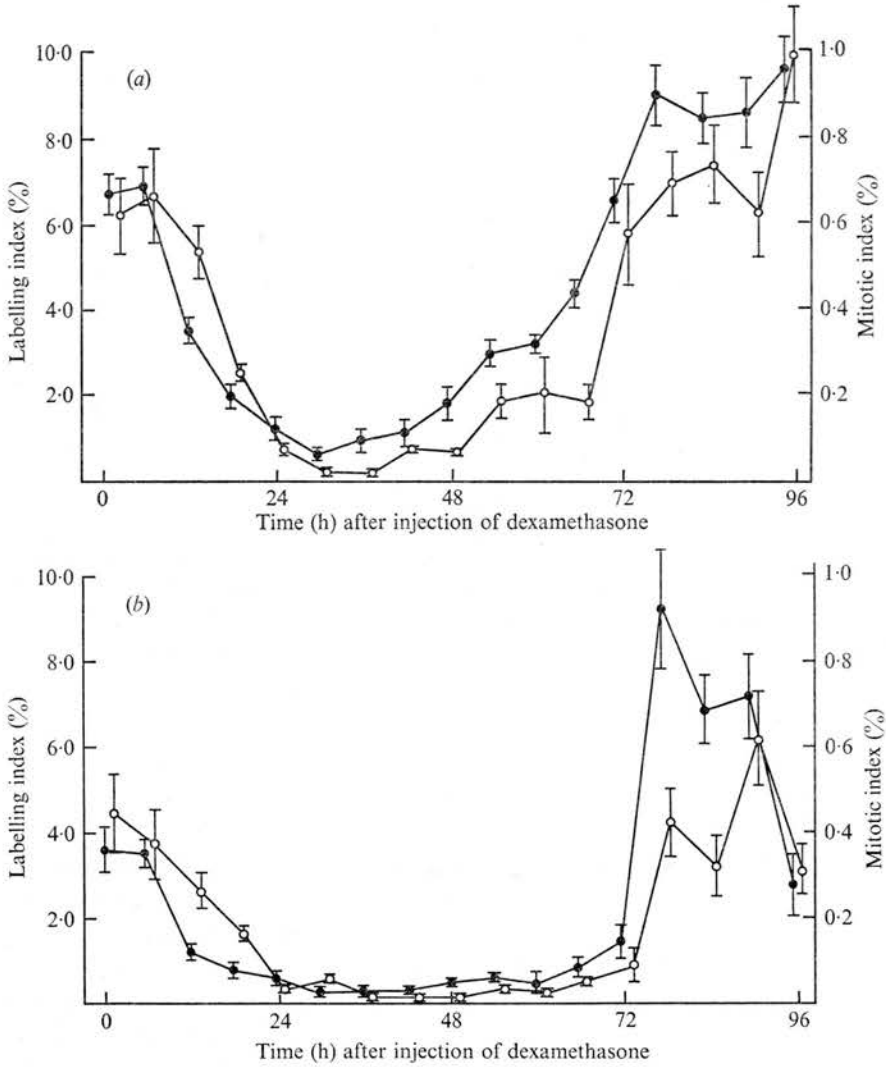

Fig. 1. Action of dexamethasone on the adrenal cortex [(*a*) zona glomerulosa; (*b*) outer zona fasciculata] of the prepubertal male rat. At time 0, animals received 3 μg dexamethasone/g body wt and the points represent the mean values for groups of three animals killed every 6 h. The labelling index (●) and mitotic index (○) show an initial depression in both zones, followed by a recovery sequence in which a considerable overshoot is apparent. Vertical lines indicate ± S.E.M.

index began to fall at about 5 h after dexamethasone administration at which point the difference in the time of fall of the two indices was much clearer and a steady decrease was apparent, with the labelling index reaching low levels by 16–27 h. In the outer z. fasciculata the initial decrease in labelling index was again apparent at 5–6 h, and a similar fall to that seen in the z. glomerulosa ensued. In both these zones the mitotic index remained relatively constant up to 13 h after dexamethasone administration, and then fell steadily, to reach very low levels by 20–21 h. Similar results were apparent in the transition zone.

In the inner portion of the gland, i.e. the inner z. fasciculata and the z. reticularis,

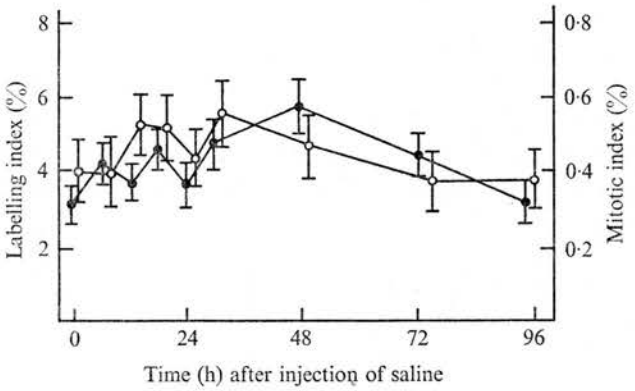

Fig. 2. Labelling (●) and mitotic (○) indices in the outer z. fasciculata of the prepubertal male rat adrenal cortex after injection of 0·9 % saline. The points indicate mean values for groups of three animals. Vertical lines indicate ± S.E.M.

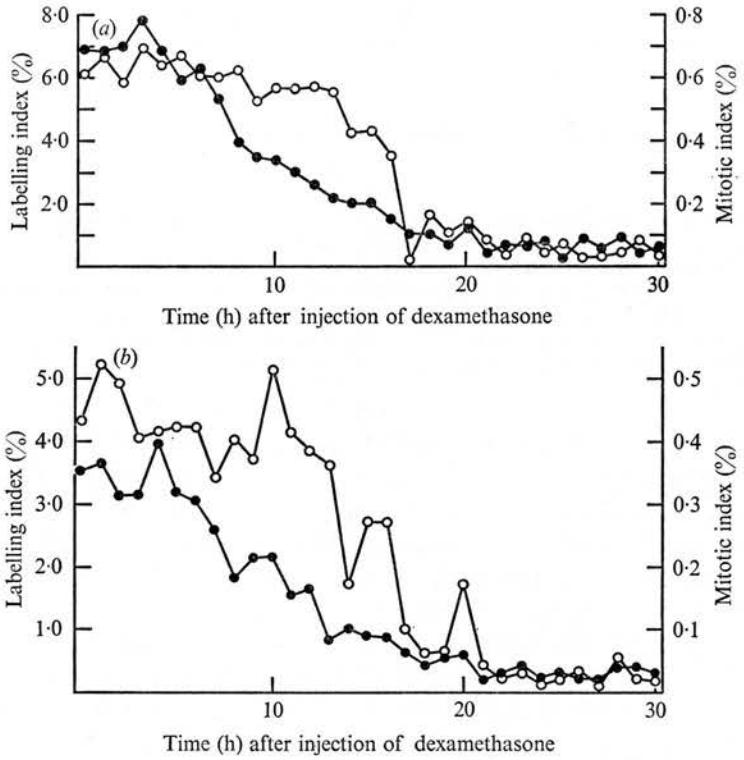

Fig. 3. Labelling (●) and mitotic (○) indices in the (*a*) zona glomerulosa and (*b*) outer zona fasciculata of the prepubertal male rat adrenal cortex after a single injection of 3 μg dexamethasone/g body wt. Each point represents the mean of two animals. In both zones the labelling index falls before the mitotic index. The scales of proliferative indices are different, so that the differential decrease in labelling index and mitotic index can be appreciated in the outer z. fasciculata.

the individual mean values from the pairs of animals studied at each hour were very variable, and no clear pattern emerged.

These results can be explained by reference to Fig. 4. In the outer cortical zones the labelling index begins to fall about 5 h after dexamethasone administration. After allowing time for absorption of the dexamethasone from the peritoneal cavity, this latent period before the fall in labelling index would suggest that dexamethasone blocked adrenocortical cells some 4–5 h before the onset of the DNA synthetic, or S, phase (see Fig. 4).

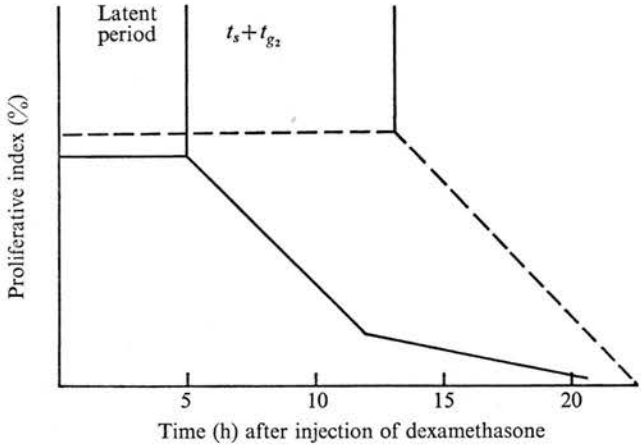

Fig. 4. An interpretation of the sequence of events after dexamethasone treatment. After a latent period of approximately 5 h, the labelling index (—) falls, to be followed by the mitotic index (– – – –) only after a further period of 8 h. Cells become blocked in the cell cycle in late G_1, 5 h before the onset of DNA synthesis, consequently 5 h after dexamethasone administration the labelling index falls. After the cells which were beyond the induced block at the time of dexamethasone injection have completed DNA synthesis and mitosis, the mitotic index falls in an additional time equal to t_s+t_{g2}, which in the outer zones of the adrenal cortex approximates to 8 h (Wright, 1971*a*).

If cells did become blocked a short time before the onset of DNA synthesis, the cells which were beyond this critical point in the cell cycle at the time of onset of dexamethasone action would be expected to progress and complete DNA synthesis, the G_2 phase and mitosis. In these circumstances, the mitotic index would remain constant for a time equal to 5 h (the initial latent period) + (t_s+t_{g2}), where t_s is the duration of DNA synthesis and t_{g2} is the duration of G_2, the pre-mitotic interval. The mitotic index would then fall as the cohort in late G_1, S and G_2 complete mitosis, and no other cells take their place because of the dexamethasone-induced block in late G_1. In the outer three zones the mitotic index fell only after 13 h. Subtracting the latent period (i.e. about 5 h), t_s+t_{g2} should approximate to 8 h. Wright (1971*b*) measured t_s and t_{g2} by the fraction of labelled mitoses technique, for the z. glomerulosa and a 'proliferative compartment', the latter being an area with high proliferative indices and corresponding to the transition zone and a portion of the outer z. fasciculata; t_s+t_{g2} for the z. glomerulosa was 8·6 h, and for the 'proliferative compartment' 9·0 h. These values support the interpretation given in Fig. 4.

Experiment 3

The results obtained in experiment 2 appear to demonstrate a block in the cell cycle in late G_1. On the other hand, Baserga, Estensen & Petersen (1965), knowing that late G_1 is a critical period in the life cycle of the cell when many factors necessary for the initiation and continuation of DNA synthesis appear, pointed out that inhibition of synthesis in late G_1 of a metabolite necessary for the continuation of DNA synthesis would force cells to stop at some point in S; cells would continue to move into S from G_1, and later become blocked in S and the labelling index would decrease, again after a latent period. The results would then be indistinguishable from a partial block of cells in G_1.

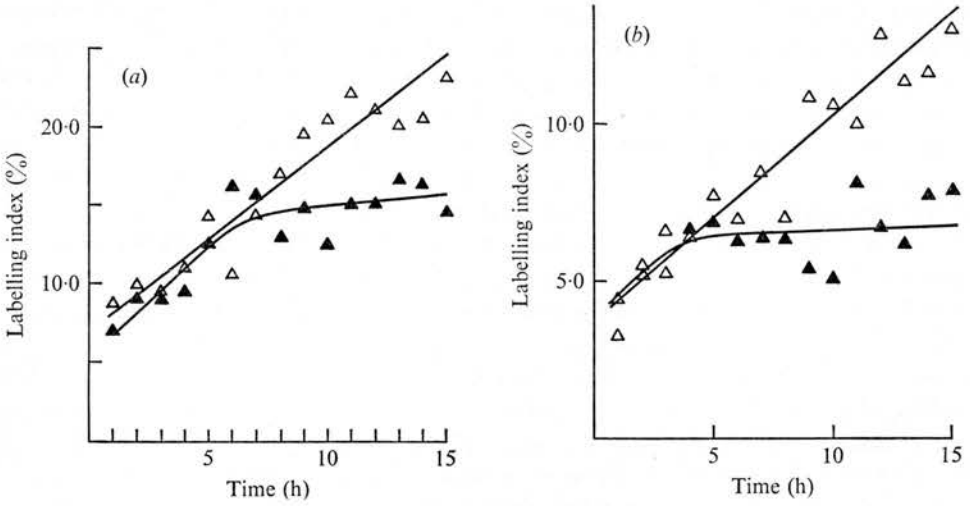

Fig. 5. Continuous labelling graphs for the z. glomerulosa (*a*) and outer z. fasciculata (*b*) of saline-treated control (△) and dexamethasone-treated (▲) experimental prepubertal male rats. Tritiated thymidine was administered at 1 h intervals after dexamethasone injection, and each point represents one animal. In the experimental animals the increase in the continuous labelling index (LI) slows after a latent period, which although variable for each zone approximates to 5–6 h. This supports the concept of a block in the flow of cells from G_1 to S. The further small rise observed in the experimental animals after the G_1–S block occurs, is due to later division of the cells which were beyond the site of the block at the time of dexamethasone injection, and which subsequently became labelled. The scales of LI are different, so that the differential rise in control and experimental animals can be seen in the outer z. fasciculata.

On the basis of the above results, dexamethasone could be blocking cells in late G_1, or acting metabolically, possibly also in late G_1, to prevent progress through the S phase. The results of experiment 3 are given in Fig. 5. Under the conditions of continuous labelling, when [^{3}H]Tdr is constantly available to cells synthesizing DNA, the increase in labelling index with time is a measurement of the rate of flow of cells from G_1 to S. If cells are being blocked in late G_1, then after the latent period of about 5 h referred to above, cells will cease to flow into S and the labelling index will cease to rise. However, if cells are becoming blocked later in S, the rate of flow of cells from G_1 to S will be unimpeded, and the rate of increase in the continuous labelling index will be close to control values. It is apparent that in the z. glomerulosa and outer z. fasciculata the labelling index in the experimental animals increased linearly

in much the same way as in the control animals over the first 5–6 h. Thereafter the rate of increase diminished, although some increase in the labelling index would be expected during this period as labelled cells, i.e. those cells which were beyond the critical point in late G_1, later divide. The results support the proposal that an actual block in late G_1 has occurred (see Discussion). Basically similar results were obtained in the remaining zones studied.

DISCUSSION

It has been shown above that dexamethasone has a profound inhibitory effect on cell proliferation in all zones of the prepubertal adrenal cortex. After a short latent period, the sequence in which the labelling and mitotic indices fell supports a block at a specific point in late G_1. The nature of the latent period will depend on the precise mechanism of dexamethasone action. If dexamethasone is acting directly on adrenocortical cells, and independent of the hypothalamus, then the latent period is made up of the time taken for dexamethasone to gain access to the adrenocortical cells, plus the interval between the specific point in late G_1 and the onset of DNA synthesis. As dexamethasone was administered intraperitoneally, the greater part of the latent period was occupied by the delay between the point of action and the S phase.

If, however, dexamethasone acts in this context on the hypothalamus the sequence of events would be quite different. After absorption, a finite time would elapse before endogenous ACTH disappeared (Berson & Yalow, 1968); adrenocortical cells deprived of ACTH are then blocked in late G_1.

The site of action in late G_1 suggests that dexamethasone is preventing one of the metabolic events at this time. Borun, Scharff & Robbins (1967) have shown that protein synthesis is necessary for the flow of cells from G_1 to S. Stein & Baserga (1972) concluded that some events in G_1 which are directly related to the onset of DNA synthesis involve gene expression. Dexamethasone may well act on one of these important events.

There was a prominent overshoot during the recovery period, the timing and magnitude of which also varied with the zone considered. Presumably the large numbers of cells which are held up in late G_1 are released into DNA synthesis, giving a sharp increase in the labelling index. Alternatively the role of endogenous ACTH in the recovery sequence should be remembered, as its reappearance could be responsible for causing the flow of G_1 to S cells to continue.

The differential rates of decrease of labelling and mitotic indices with time after dexamethasone administration (Fig. 3) show a similar format in the outer three zones, but because of the variation between individual readings in the z. reticularis and outer z. fasciculata, no clear pattern emerged.

The precise mechanism leading to the production of a G_1 to S block in the cell cycle of adrenocortical cells after dexamethasone treatment remains to be explained: a mechanism involving ACTH, or a direct action by dexamethasone. Dexamethasone induced a G_1 to S block in the cells of the z. glomerulosa, an area known to remain intact after hypophysectomy and therefore presumably not dependent upon ACTH for the maintenance of its structural integrity (Deane & Greep, 1946). Wright (1971*b*) and Wright *et al.* (1973) have shown that in the prepubertal animal much cell production takes place in the z. glomerulosa, followed by migration of these cells to the

inner zones. It is difficult to reconcile this concept of adrenocortical cytogenesis with the absence of ACTH control of cell proliferation in the z. glomerulosa, but the recent observations of Shire & Stewart (1972) have shown that at least in the mouse ACTH has a modifying effect on the morphology of the z. glomerulosa. The findings of Palkowitz & Mitro (1968) indicate that in prepubertal rats of this age an action of dexamethasone mediated by the hypophysial-pituitary-adrenal system is possible, and that depression of endogenous ACTH may play a part in the response.

A direct action of dexamethasone on adrenocortical cells is difficult to disprove. The site of action, i.e. in late G_1, is very similar to results obtained in many cell types with other glucocorticoids. For example, in mouse lymphoid cells hydrocortisone prevents cells from entering the S phase (Epifanova, 1971), and Frankfurt (1968) has shown that a single injection of hydrocortisone inhibits the onset of DNA synthesis in the squamous cells of the mouse forestomach. The findings of Lahtiharju, Rasanen & Tier (1964), studying the action of glucocorticoids on cell proliferation in the small bowel mucosa, may be interpreted in a similar manner. Glucocorticoids are also known to prolong the cell cycle in HeLa cells (Kollmorgen & Griffin, 1969) and in mouse small bowel epithelium (Laguchev, 1969) by an action on G_1. The similarity of these results to the present observations suggests that dexamethasone acts directly on adrenocortical cells with the production of a similar flow block.

Mention should be made of a report from Ueberberg, Stöcker & Städtler (1970) which concluded that dexamethasone had no effect on adrenocortical cell proliferation. These workers used older animals, and it is difficult to reconcile these observations with the present results. However, the present data were obtained from the adrenal cortex in a phase of active growth (Wright *et al.* 1973) and the conclusions may indeed not apply to the adult situation.

It is concluded that the depressive effect of dexamethasone is most probably a direct effect on adrenocortical cells, although an action on endogenous ACTH cannot be excluded. In spite of this qualification, the results show that a steroid-sensitive step does exist in adrenocortical cells. The G_1 to S transition is an important phase in mammalian cells, currently regarded as a point at which the control of cell proliferation is exerted. It is possible that ACTH may also act in a stimulatory or permissive manner at this point; if physiological steroids have a similar action to dexamethasone, i.e. inhibition of adrenocortical cell proliferation, then cell proliferation in the adrenal cortex may be controlled by the relative stimulatory and inhibitory effects of endogenous ACTH and adrenocortical steroids, the plasma levels of which are themselves closely related.

We thank Professor A. G. Heppleston for continual encouragement and advice. Miss Doreen Hardy and Mr and Mrs H. Elliott rendered skilled technical assistance and Mrs M. Heslop typed the manuscript. This work was performed with the aid of a grant from the North of England Branch of the Cancer Campaign.

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The measurement of the cell cycle time in squamous epithelium using the metaphase arrest technique with vincristine

65

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Accepted for publication 1 November 1976

SUMMARY

In squamous epithelia with a single layer of germinative cells, the age distribution of cells in the cell cycle is shown to depend on the direction of the mitotic axis (i.e. a line joining the nuclei of daughter cells) relative to the plane of the basal layer. When axes are in the plane of the basal layer the age distribution is exponential; when cells divide at right angles to the plane of the basal layer, the age distribution is rectangular. When there is a ratio of vertical to horizontal axes, the age distribution is intermediate but can be calculated from knowledge of the proportion of axes in the plane of the layer. Squamous epithelia can be classified according to this arrangement of axes.

When there are multiple layers of germinative cells, as in psoriasis, the age distribution is shown to be exponential to a good approximation, whatever the direction of the mitotic axes in the several layers.

The importance of these observations is demonstrated by analysing metaphase arrest experiments with vincristine in the single layer of germinative cells in the mouse oesophagus, and in the several layers found in psoriatic epidermis. Choice of the wrong age distribution leads to an error of 6 h in the oesophagus and 23 h in psoriatic epidermis, when the mean cell cycle time is calculated.

It is concluded that, in squamous epithelium, it is most important to know the age distribution before calculating the cell cycle time by methods involving measurement of the rate of entry of cells into mitosis or DNA synthesis.

The current interest in the cell population kinetics of the epidermis has resulted in many reported studies using autoradiographic methods with tritiated thymidine (e.g. Weinstein & Frost, 1968) and metaphase arrest techniques with such agents as colchicine or colcemid (e.g. Fisher & Wells, 1968).

Particularly in these epidermal studies, few authors have paused to consider the assumptions upon which their calculations are based. The most important of these affects both autoradiographic and metaphase arrest experiments, and this is the *age distribution* of cells in the cell cycle. In both normal

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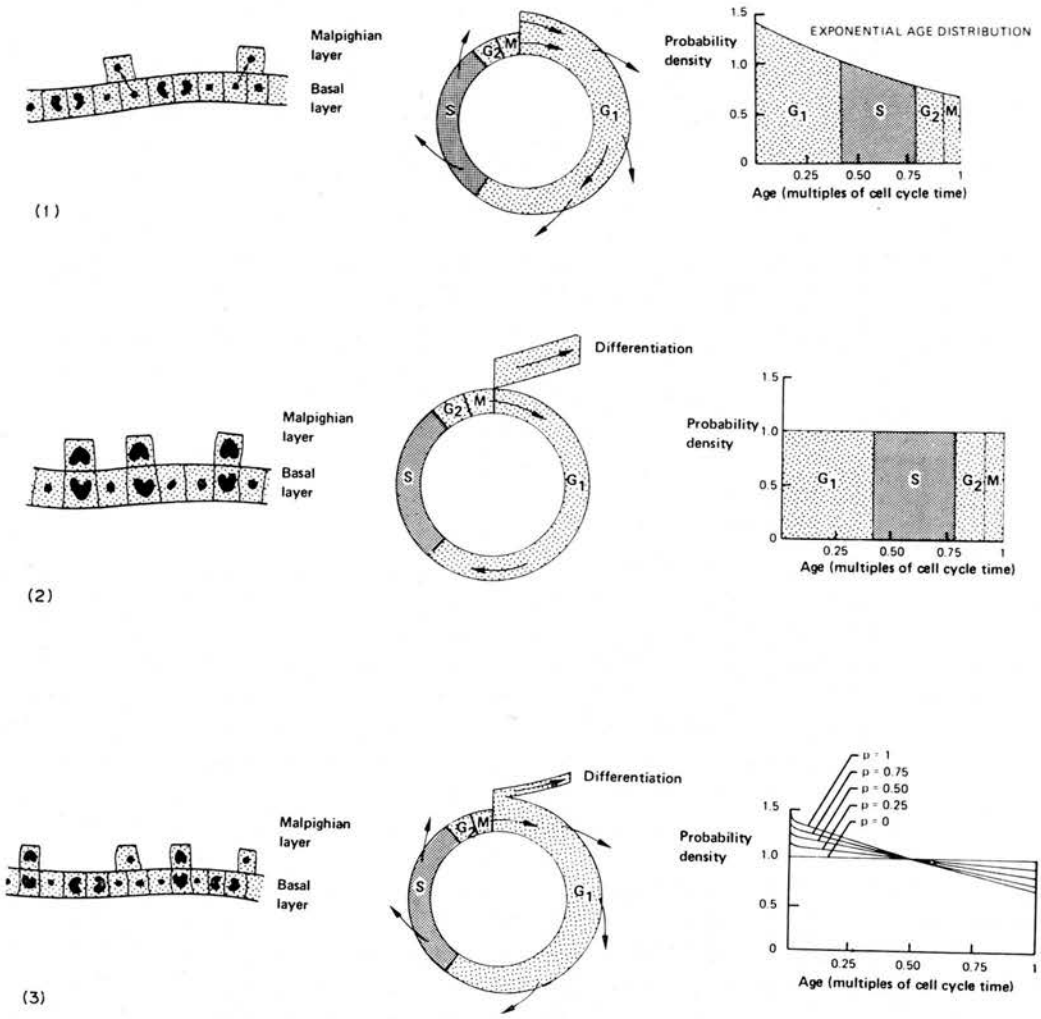

FIGURE 1. The situation in an epithelium with a single layer of germinative cells, where mitotic axes are horizontal, and cells divide in the plane of the basal layer (e.g. mouse epidermis). Cells are not lost from the germinative layer at mitosis, but cell loss into the Malpighian layer is at random throughout the remainder of the cell cycle (indicated by the arrows). The resulting age distribution is then exponential.

FIGURE 2. An epithelium with a single layer of germinative cells, where all mitotic axes are vertical. Hence at each mitosis one cell is lost into the Malpighian layer and the age distribution is rectangular.

FIGURE 3. A single layer of germinative cells where there are both vertical and horizontal axes. At division, only a fraction of mitoses donates one cell to the Malpighian layer; the remaining mitoses supply two new cells to the germinative basal layer, and in these cells migration will again be random throughout the cell cycle (indicated by the arrows). The resulting age distribution will depend upon p, the proportion of cells with horizontal mitotic axes. The age distributions are drawn for different values of p.

human epidermis and psoriatic epidermis incorrect assumptions have been made concerning the age distribution of proliferating cells (Epstein & Maibach, 1965; Weinstein & Frost, 1968; Goodwin, Hamilton & Fry, 1974).

In terms of cell production, squamous epithelium is usually considered to be in a steady state; the number of cells born in basal layers equals the number of cells lost from the surface, and there is no net increase in cell number. If, at each mitosis, one cell remains in the cell cycle and one leaves the cycle and differentiates, cell number is kept constant, and the numbers of cells in any phase of the cell cycle is proportional to the duration of that phase. The age distribution is then said to be *rectangular*. This is the distribution that is usually assumed to apply to squamous epithelium with a single proliferating layer (such as the rodent oesophagus and some types of epidermis), and also to psoriasis, where up to three layers of basal germinative cells are found. It will be shown that this last assumption is wrong, and introduces serious errors into calculations of the cell cycle time in these tissues.

Previous applications of the metaphase arrest method to epidermis have incurred errors and criticism due to suspect technique (Fisher & Wells, 1968; Fisher, 1968); the metaphase arrest technique will be shown to yield useful information only if the limitations of the method are fully appreciated.

THEORETICAL CONSIDERATIONS

The age distribution in squamous epithelium

In the calculation of the age distribution in basally situated proliferating cells, the direction of the mitotic axis is a most important consideration. The axis may be horizontal, and cells divide in the plane of the basal layer; or vertical, in which case cells divide perpendicularly to the plane of the basal layer.

Consider a single layer of proliferating cells (Fig. 1). There are several possible situations:

(i) Here *all* basal cells divide with the mitotic axis in the plane of the basal layer. The two young cells remain within the basal layer, and an adjacent cell is ejected (assumed to be *random* with respect to age in the cell cycle) to differentiate. There will then be a preponderance of young cells in the basal layer, and the population density will fall with age in the cell cycle as basal cells undergo the random removal process.

The age distribution is then *exponential*, and the probability $P(\tau)$ that a cell will be at age τ is

$$P(\tau) = 2\,\frac{\log_e 2}{T_c}\exp\left[-\frac{\log_e 2}{T_c}\tau\right] \qquad (1)$$

where T_c is the cell cycle time (Steel, 1968).

This distribution is shown in Fig. 1. At each mitosis the two young cells remain in the basal layer, and the cell cycle; the probability of these cells then continuing in cycle then declines exponentially with age in the cell cycle. This kinetic situation, with all axes horizontally orientated, is found in the squamous epithelium of the hamster cheek pouch (Brown & Berry, 1968) and also in the epidermis of mature mice (Smart, 1970a).

A random removal process for basal cells is supported by the work of Marques-Periera, Greulich & Leblond (1963) in the mouse oesophagus, and also by Brown & Berry (1968) in the hamster cheek pouch. Iversen, Bjerknes & Devik (1968) have suggested that in normal squamous epithelium cells in S, G_2 and M do not migrate; basal cell removal would then be random in G_1 but because $t_s + t_{g2} + t_m$ is short with respect to T_c (Brown & Berry, 1968; Hegazy & Fowler, 1973), from the point of view of the age distribution, random removal in G_1 is effectively the same as random removal throughout the cell cycle.

(ii) In this case, all mitotic axes are perpendicular to the plane of the basal layer. At each mitosis,

one cell remains to proliferate in the basal layer, and the other migrates (Fig. 2). There is then a true *rectangular age distribution*. The probability of finding a cell at age τ is then

$$P(\tau) = \frac{1}{T_c} \tag{2}$$

Here the fraction of cells in any phase is proportional to the length of that phase. This kinetic situation is very rare, but has been reported in embryonic mouse epidermis (Smart, 1970a).

(iii) This is a more usual situation (Fig. 3) where mitotic axes are found in both planes, in varying proportions; some axes are vertical with immediate loss of one cell into the Malpighian layer, and some are horizontal, with retention of both young cells.

To calculate the age distribution, Equation (1) must be modified to incorporate p, the proportion of mitoses with horizontal axes, and the probability of finding a cell at age τ is then

$$P(\tau) = \frac{1+p}{p}\frac{\log_e(1+p)}{T_c}\exp - \left[\frac{\log_e(1+p)}{T_c}\tau\right] \tag{3}$$

Fig. 3 illustrates the age distribution; at each mitosis the fraction of vertical axes contributes one cell to the Malpighian layer and one cell to the basal layer, while those mitoses with horizontal axes contribute both cells to the basal layer. Fig. 3 also shows the age distribution of cells with p set at several values. With $p = 1$, the age distribution is exponential, and the initial ordinate falls with decreasing values of p until, when $p = 0$, the age distribution is again rectangular.

This more complex arrangement, with a distribution of mitotic axes, is seen in many squamous epithelia, for example normal human epidermis (Pinkus & Hunter, 1966).

(iv) In hyperplastic conditions more than one layer of germinative cells may be present (Bullough, 1972); in psoriasis there can be up to three layers of germinative cells (Penneys *et al.*, 1970).

In this case most germinative cells will be retained within the population after division, and there will be a preponderance of young cells; an exponential form of age distribution would thus be anticipated. In psoriatic epidermis mitoses show both vertical and horizontal axes, but there are two limiting cases. If all mitoses have horizontal axes then all young cells are retained after mitosis; it can be shown that

$$P(\tau) = \frac{2\log_e 2}{T_c}\exp - \frac{\log_e 2}{T_c}\tau \tag{4}$$

i.e. the age distribution is exponential.

At the other exteme is the situation where all mitotic axes are horizontal to the plane of the basal layer. The limiting value in Equation (3) is the probability P_1 of finding a cell at age τ in the first layer of germinative cells,

$$P_1(\tau) = \frac{1}{T_c}$$

i.e. in the basal layer, a rectangular age distribution obtains. In the second layer, the age distribution is

$$P_2(\tau) = \frac{1{\cdot}58}{T_c}e^{-\tau/T_c} \tag{5}$$

In the third layer

$$P_3(\tau) = \frac{1}{T_c}\{2{\cdot}72\,e^{-\tau/T_c} - 1{\cdot}43\,e^{-1{\cdot}58\,\tau/T_c}\} \tag{6}$$

These distributions are shown in Fig. 4; even in this extreme case the overall age distribution is close to exponential. Since axes appear to be distributed between vertical and horizontal in psoriasis, an addition of the individual distributions will be close to exponential.

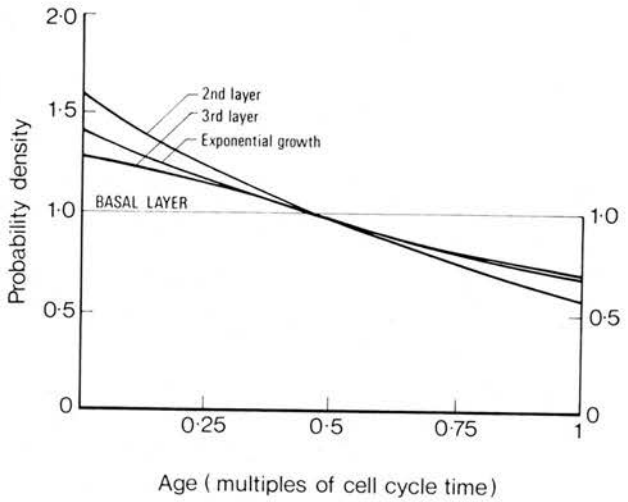

FIGURE 4. Age distributions drawn for three layers of germinative cells, where all mitotic axes are vertical. In the first layer, (1) the age distribution is rectangular, but in the second (2) and third (3) layers, the age distribution is of an exponential form, and an addition of the three distributions would also give an exponential type of age distribution.

We conclude that, in squamous epithelia where multiple layers of germinative cells are found, the age distribution for the proliferative cells as a whole will always be close to exponential; it should however be noted that this is a theoretical argument at present, but amenable to experiment.

MATERIALS AND METHODS

Experiment 1

Seventeen male A_2 G mice aged 7–8 months were given vincristine sulphate (Oncovin, Lilly) in a dosage of 1 mg/kg body weight by intraperitoneal injection at 09.00 h. As part of a separate experiment the animals had been treated with urethane 14 weeks previously. Animals were killed by cervical dislocation at 0·25 h intervals for a period of 4·25 h. Oesophagi were removed and fixed in Carnoy's fluid for 6 h, and embedded in paraffin. Transverse sections, cut at a thickness of 5 μm, were stained with Harris's haematoxylin. The metaphase index was determined by counting at least 2000 basal nuclei in each specimen.

Five mice were also killed at 09.00 h, and oesophageal sections prepared. In these, 155 mitoses were examined for the determination of the direction of mitotic axes, using anaphases and telophases.

Experiment 2

To determine the optimum dose of vincristine in psoriasis, patients with *stable* psoriatic plaques on the lower back were given increasing doses of vincristine by intradermal injection. A 5/8 in 25 gauge needle was inserted into the uninvolved skin close to the plaque, and the tip was advanced to lie under the site in the plaque to be biopsied. The bevel was sited upwards by placing a mark on the hub of the needle. Doses of vincristine varying from 0·01 to 20 μg were given.

At exactly 2·5 h later a biopsy was taken from each injected site, using a 3 mm punch and 2% lignocaine as local anaesthetic. The biopsies were fixed in Carnoy's fluid, sectioned at 4 μm, and stained with Harris's haematoxylin. In each specimen the metaphase index was found by counting at least 6000 germinative cells. The metaphase index was then plotted against log dose of vincristine.

Experiment 3

As a result of Experiment 2, eight patients were given 1 μg of vincristine into three separate sites in the periphery of a stable psoriatic plaque, and biopsies were taken at 0·75, 1·5 and 2·5 h after injection. A biopsy was also taken at 0 h for determination of the native mitotic index. Sections were prepared and the metaphase index in germinative cells was determined as described in Experiment 2. The metaphase index was then plotted against time after injection of vincristine.

The method of fitting the metaphase accumulation line will depend on the age distribution of the cells being considered. If, for example, in the basal layer of the oesophagus all mitotic axes were vertical, ($p = 0$), and there was a rectangular age distribution, the line fitted would be

$$M(t) = \frac{1}{T_c}(t_m + t) \tag{7}$$

where $M(t)$ is the metaphase index at time t, and t_m is the duration of mitosis.

However, if all mitotic axes were horizontal, ($p = 1$) and the age distribution exponential, the line fitted would be

$$\log_{10}\{1 + M(t)\} = \frac{\log_{10}2}{T_c}(t_m + t) \tag{8}$$

$\log_{10}\{1 + M(t)\}$ being the collection function of Puck & Steffen (1963).

When we have both horizontal and vertical axes (p lies between 0 and 1), then we fit

$$\log_{1+p}\{1 + p\,M(t)\} = \frac{1}{T_c}(t_m + t) \tag{9}$$

In each case the best fit was obtained by the method of least squares.

RESULTS

(i) *Single layer of germinative cells—the mouse oesophagus*

In the uninjected animals, 155 mitoses were suitable for determination of the direction of the axes; 58 had horizontal axes, 82 had vertical axes, while 15 axes were intermediate and have been treated as if they were vertical, since one of the basal cells presumably left the basal layer. Thus $p = 0{\cdot}37$ ($\pm 0{\cdot}04$, s.e.).

Fig. 5 shows the metaphase accumulation graph after vincristine injection in the basal layer of the mouse oesophagus. In the readings prior to 75 min, telophases were present and it was evident that vincristine could not have taken full effect; to avoid an overestimate of the cell cycle time, these observations were excluded from the calculation.

In Fig. 6 the line is fitted using Equation (9), with p set at 0·37; this gives a mean cell cycle time of 43 h with 95% confidence limits of 32 and 67 h.

(ii) *Multiple layers of germinative cells—psoriasis*

In Fig. 6 the 2·5 h metaphase index in psoriatic plaques is plotted against the log dose of vincristine. With increasing doses of vincristine the index rises to a peak at 1 μg; there is a fall with larger doses. We conclude that the optimal dose is 1 μg, injected intradermally.

Fig. 7 shows the metaphase accumulation line after vincristine for the patients with psoriasis. After 0·75 h there is apparent linearity of metaphase collection. It has been shown above that, with multiple layers of germinative cells, the age distribution will be exponential, and so the line fitted is Equation (8). In the biopsies taken at 0 time, a count of 100 suitable mitoses showed that 58% were

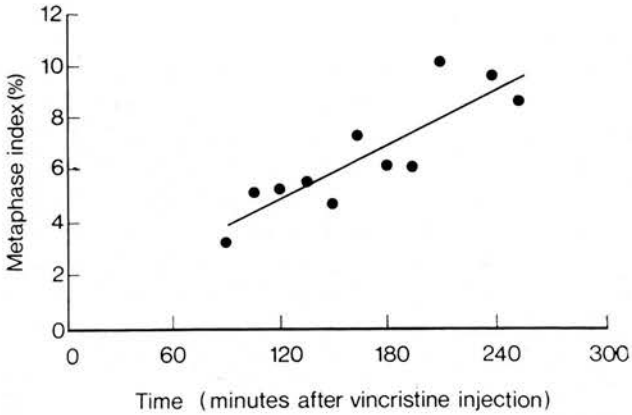

FIGURE 5. Metaphase accumulation in the mouse oesophagus plotted against time after vincristine injection. Vincristine is fully active at 75 min after injection, and metaphase accumulation is linear thereafter. The line is fitted using Equation (9).

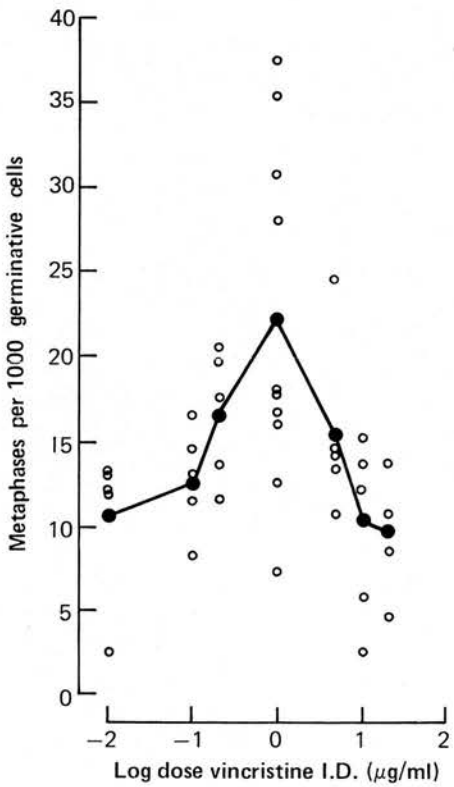

FIGURE 6. The metaphase index in psoriatic plaques at 2·5 h after intradermal injection of various doses of vincristine. Maximum values are apparent with 1 μg.

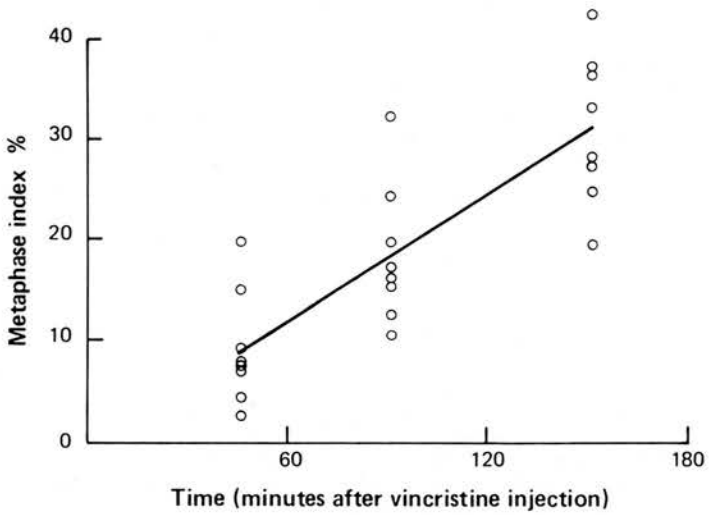

FIGURE 7. Metaphase accumulation in psoriatic plaques of 8 patients after intradermal injection of 1 μg of vincristine. Here vincristine becomes fully active at 45 min and again metaphase accumulation is linear thereafter. The line is fitted using Equation (8).

vertical, 24% were horizontal, and 18% were intermediate. Although these were not separated into layers, it is apparent that neither of the two extreme cases described above apply.

Equation (8) gives a mean cell cycle time of 56 h with 95% confidence limits of 39 and 97 h. If a rectangular age distribution were used, and Equation (7) applied, a mean cell cycle time of 79 h would be obtained.

DISCUSSION

It is evident that, in squamous epithelia with only one layer of germinative cells, the *age distribution depends on the direction of the mitotic axes*. Where all axes are horizontal the age distribution is *exponential*; according to Smart (1970a) this is the situation in the epidermis of the mature mouse. A rectangular age distribution is rare, but may occur in neonatal mouse epidermis, where all mitotic axes are vertical (Smart, 1970a). Where there are both vertical and horizontal axes, the distribution is modified but can be calculated. For example, Pinkus & Hunter (1966) determined the direction of the mitotic axis in human epidermis and using the notation described in Fig. 1 reported 34% horizontal, 54% vertical and 12% intermediate. This means that the age distribution in human basal cells is not rectangular, as assumed by most workers, and consequently the cell cycle time calculated by Weinstein & Frost (1968) of 435 h, assuming a rectangular age distribution, will be shortened.

The present results indicate that the basal layer of the oesophagus also has both horizontal and vertical axes, with an intermediate age distribution. If this fact is not considered, and a rectangular distribution applied, an error of about 6 h is made in the mean cell cycle time as measured by metaphase arrest methods. Obviously the same consideration applies when calculating a cell cycle time from t_s and the flash labelling index. Previous data on mitotic axes in mouse oesophagus is conflicting; Marques-Periera *et al.* (1963) reported that 96% of axes were horizontal whereas the observations of Smart (1970b) are similar to ours; he observed 66% horizontal, 23% vertical and 10% intermediate in adult mice.

The present considerations may explain an anomaly reported by Hegazy & Fowler (1973) in mouse epidermis. In the equation

$$I_s = \mu I_p t_s / T_c \quad (10)$$

μ is a constant, defined by Steel (1968) which for an exponential age distribution should equal 0·73; in fact, μ was calculated at 0·92, 1·28 times too large. This discrepancy could be explained by a fraction of cells with vertical axes lowering the initial ordinate on the exponential age distribution.

The results for the multiple layers of germinative cells in psoriasis are particularly interesting. *The age distribution is exponential, and not rectangular as assumed by previous workers.* The mean cell cycle time is 56 h, but there is a considerable spread of individual cell cycle times; these observations have been confirmed by fraction of labelled mitoses studies (Duffill, Wright & Shuster, 1976). Even so, there would be an error of 23 h in the mean cell cycle time if the wrong age distribution was chosen. In these circumstances, the adoption of the correct age distribution is important; for example, Goodwin *et al.* (1974) calculated a cell cycle time of 91 h in psoriasis, using a rectangular age distribution. When the correct exponential age distribution is applied, this value reduces to 62 h, in line with the present estimate.

Most authors conclude that basal cells are removed by a random process (Brown & Berry, 1968; Marques-Periera *et al.*, 1963; Hegazy & Fowler, 1973). However, Iversen *et al.* (1968) suggested that, with all mitotic axes vertical, in mouse epidermis, the oldest G_1 cell nearest a mitosis migrated. The evidence for this conclusion was not strong, and depended only on a better fit to continuous labelling data than was obtained with a random removal process.

Weinstein (1975) has emphasized the difficulties in defining 'germinative cells' in psoriasis. We have used the criteria described by Weinstein & Frost (1968). In the determination of the direction of the mitotic axes, only mitotic figures where the axis was clearly defined, i.e. anaphases and telophases, were assessed.

Both in the oesophagus and psoriasis we have calculated a 'cell cycle time'; strictly speaking, this is only an *apparent* cell cycle time, since we do not know the size of the growth fraction. If the growth fraction was less than 1, then this would reduce the calculated value for the cell cycle time. In squamous epithelia with a single layer of germinative cells there is evidence that the growth fraction is unity (Hegazy & Fowler, 1963) but in psoriatic epidermis the evidence is not good. The assertion by Weinstein (1971) that all germinative cells are proliferating, because all are labelled at the end of semicontinuous application of methotrexate for 36 h is suspect, since any non-cycling cells would be brought into cycle by the cell injury and death produced by the methotrexate.

The form of the dose–response curve in psoriasis is very similar to that reported for vincristine by Tannock (1967) who applied the drug to rat small bowel, and a transplantable rat tumour. There was a rise in metaphase index to an optimum dose, and then the index fell with increasing high doses of vincristine; this latter effect is possibly due to toxic action on dividing cells.

The present results show how important it is to ensure that linearity of metaphase arrest occurs in these experiments. In both tissues studied a delay period was apparent, as has been described in other tissues (Al-Dewachi *et al.*, 1975). If this delay were not excluded the cell cycle time would be overestimated. It is also important to keep the collection period short to avoid errors due to metaphase degeneration (Aherne & Camplejohn, 1972). Thus it is almost certain that Fisher (1968) incurred errors due to metaphase degeneration. Further, in this work there was no attempt to establish linearity of metaphase accumulation or to exclude any delay before the agent becomes fully operative. In the present work it should be noted that, in each case, the confidence limits of the cell cycle time are large. However, in the oesophagus the wrong age distribution leads to an error of 6 h, and in psoriasis to an error of 23 h, and it is worthwhile trying to eliminate this error.

The experimental mice were obtained for a separate experiment on urethane-induced pulmonary carcinogenesis. There was no evidence of any histological effects on the oesophageal epithelium,

but of course the urethane may have affected the cell cycle time. However, this should not interfere with the general conclusions which we have drawn concerning age distribution in squamous epithelia, and its effect on the determination of kinetic parameters.

These conclusions are: (1) With a single layer of germinative cells, the age distribution depends on the direction of the mitotic axes, and may be calculated. (2) With multiple layers of germinative cells, as in psoriasis, the age distribution is exponential. (3) It is important that these considerations be incorporated into calculations of the cell cycle time.

ETHICAL CONSIDERATIONS

All patients were given a full explanation concerning the procedure before informed consent was obtained. The procedures were passed by the Ethical Committee of the Newcastle-upon-Tyne University Hospitals Group.

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Cell Tissue Kinet. (1977) **10**, 429–436.

AN *IN VIVO* STATHMOKINETIC STUDY OF CELL PROLIFERATION IN HUMAN GASTRIC CARCINOMA AND GASTRIC MUCOSA

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(*Received* 7 *May* 1976, *revision received* 20 *December* 1976)

ABSTRACT

Cell production rates were studied in a group of eleven patients with carcinoma of the stomach using an *in vivo* technique with vincristine. In normal pyloric mucosa estimates ranged from 8 to 15 cells/1000 cells/hr, and in the carcinomas from 3 to 24 cells/1000 cells/hr. Because of the very large variation in the data, comparison between individual tumours and normal mucosa is not regarded as being worthwhile at this stage. The implications of these results for previous human *in vivo* stathmokinetic experiments are also discussed.

The special relevance of cell population kinetics in human tumours is to radiotherapy and cytotoxic chemotherapy; the ultimate aim of oncologists is to be in a position where kinetic parameters in individual human tumours can be measured and used to predict therapeutic responses to a specially designed radiotherapeutic and chemotherapeutic regimen. Viewed in this light, experiments involving tritiated thymidine (^{3}H-TdR) would appear to be of strictly limited usefulness. The technique is time consuming in terms of both technical and quantitative procedures, and a delay of 6–8 weeks in obtaining data which might be needed quite urgently has prompted investigators to seek an alternative, more rapid technique.

One of these is the stathmokinetic or metaphase arrest method, in which routine (but thin) paraffin sections can be used, and the method of quantitation merely involves the determination of mitotic indices. The amount of kinetic information is limited when compared with the more sophisticated ^{3}H-TdR methods, but one useful kinetic parameter which should be available is the birth rate (k_b) read directly from the slope of the mitotic accumulation graph. The birth rate can be converted to a cell production rate, expressed as cells per 100 or 1000 per hour (Smith, Thomas & Riches, 1974). The potential doubling time (t_{pd}) can be calculated from the birth rate. From data of this type it might be expected that one could distinguish between slowly and rapidly proliferating tumours, in order to predict which neoplasms would reasonably be expected to respond to radiotherapy or chemo-

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therapy. Further, the proliferative rate of the tumour may be compared with that of the native, adjacent tissue of origin, to obtain some idea of the differential rate of cell killing to be expected from radiotherapy.

Possibly with these thoughts in mind, several groups of workers have endeavoured to apply stathmokinetic techniques to a variety of human tumours. Using colcemid, Refsum & Berdal (1967) reported a mean t_{pd} of 2·6 days in a group of sixty-one tumours, mainly carcinomas of the mouth and throat; Fulker, Cooper & Tanaka (1971), in vesical carcinoma, found that t_{pd} varied from a mean of 22·2 days in well differentiated tumours down to 2·2 days in undifferentiated carcinomas, and there was a clear negative correlation between the degree of differentiation and the rate of cell proliferation. Also with colcemid, Aherne & Buck (1971) reported a mean t_{pd} of 4·2 days in a group of five neuroblastomas, and for a group of six nephroblastomas 7·8 days.

The vinca alkaloids have also been employed; Meyer & Donaldson (1969) used vinblastine to measure t_{pd} which varied between 6 and 9 days in oral squamous carcinomas, while Camplejohn, Bone & Aherne (1973) biopsied patients with carcinoma of the rectum before and after vincristine, and reported a mean t_{pd} of 192 hr; further, Camplejohn *et al.* considered that cell production rates were lower in the tumours than in the adjacent, normal mucosa.

Whilst these data seem useful and interesting at face value, in spite of apparently good linearity of metaphase collection, the number of readings in individual cases in most studies is small; although 95% confidence intervals for t_{pd} have not been calculated, these are probably considerable.

In this paper we report studies made of eleven patients with carcinoma of the stomach, employing a stathmokinetic technique with vincristine. The adjacent normal mucosa, and hyperplastic and metaplastic gastric mucosa were also studied. Although at first sight the data appear reasonable and indeed useful, closer inspection shows how difficult it is to draw worthwhile conclusions with this technique in its present form.

MATERIALS AND METHODS

All patients received full information concerning the experimental procedures, and gave informed consent. The experimental procedure was submitted and passed by the Ethical Committee of the Newcastle upon Tyne University Hospitals Group. As a first step, during diagnostic endoscopy, biopsies were taken of the gastric mucosa, and also of the pyloric and fundal mucosa. These biopsies were fixed in 10% neutral buffered formol saline and post-fixed in formol-corrosive. Routine paraffin sections were used to establish and report the diagnosis of malignancy. Once diagnosis was established, serial sections were cut at a thickness of 3 μm for mitotic counting, and stained with haematoxylin and eosin.

Immediately prior to surgery, when the patient was anaesthetized, vincristine sulphate (Oncovin, Lilly) was infused rapidly via an intravenous cannula at a dose of 0·045 mg/kg body weight (Wright *et al.*, 1973). A note was made of the time the drug was given. When the stomach was exposed a gastrotomy incision was made, and biopsies of the tumour, and of normal gastric mucosa, were made with pituitary rongeurs, care being taken to biopsy the same portion of the tumour in each case, as far as was possible. Thereafter, during mobilization of the stomach, biopsies were taken at intervals which were convenient to the operative procedure. As far as possible, the period after the blood supply to the stomach had

been seriously compromised was avoided, although during the resection procedure it was inevitable that some decrease occurred in blood supply to the stomach.

In the tumour material, at least 2000 nuclei were counted from each biopsy, and the mitotic index determined for each time period. In the adjacent normal mucosa, considerable difficulty was experienced in endoscopy material in obtaining sufficient well-orientated gastric glands in which all cells in the neck, isthmus and pit could be counted to collect 2000 nuclei in each biopsy. In mucosae showing intestinal metaplasia, as far as possible all cells in the 'crypt' were included in the count.

The results were plotted as mitotic index against time after vincristine, and where appropriate a line was fitted to the data, using least squares and assuming a rectangular age distribution (Wright, Morley & Appleton, 1972). For comparison, the data were also analysed assuming exponential conditions, using the relationship

$$\log_2 (1 + I_m) = \frac{1}{t_{pd}} (t_m + t) \qquad (1)$$

Where only two biopsies (before and after vincristine) were available or suitable for counting, then the rate of entry into mitosis, and hence the birth rate, was calculated from the difference between the mitotic index of the two biopsies. Otherwise the birth rate was calculated from the regression line. From this birth rate value, the cell production rate can be conveniently expressed in cells produced per 1000 cells per hour.

All the cases studied were adenocarcinomas of the stomach of varying degrees of differentiation.

RESULTS

Gastric carcinoma

Three cases in the series were excluded because of the presence of anaphases in the post-vincristine biopsies. In ten of the eleven cases studied more than two time periods were available, and the results are shown in Fig. 1. The lines are fitted for a rectangular age distribution. The cell production rates with 95% confidence intervals are given in Tables 1 and 2 together with potential doubling times also with 95% confidence intervals; these are calculated assuming that the errors conform to a Gamma distribution.

It is apparent that in most cases the confidence limits for the parameters estimated are extremely wide; in several instances the lower limit for the cell production rate is negative; the lower confidence limit for the line is so wide that a line of negative slope could be drawn. Consequently the upper confidence limit for t_{pd} is infinite.

The estimates for the cell production rates range from 3·4 to 24·3 cells/1000 cells/hr. In five of the ten cases the lower confidence limit was infinite, and it is difficult to know how to interpret these estimates; it is much more realistic to quote the confidence interval rather than the mean value.

In several cases the correlation coefficient is quite high, even over 0·9, but the confidence limits remain wide. This is because of the small number of time intervals available for study (see Discussion).

The data do not provide much information about the absolute or relative rates of growth of

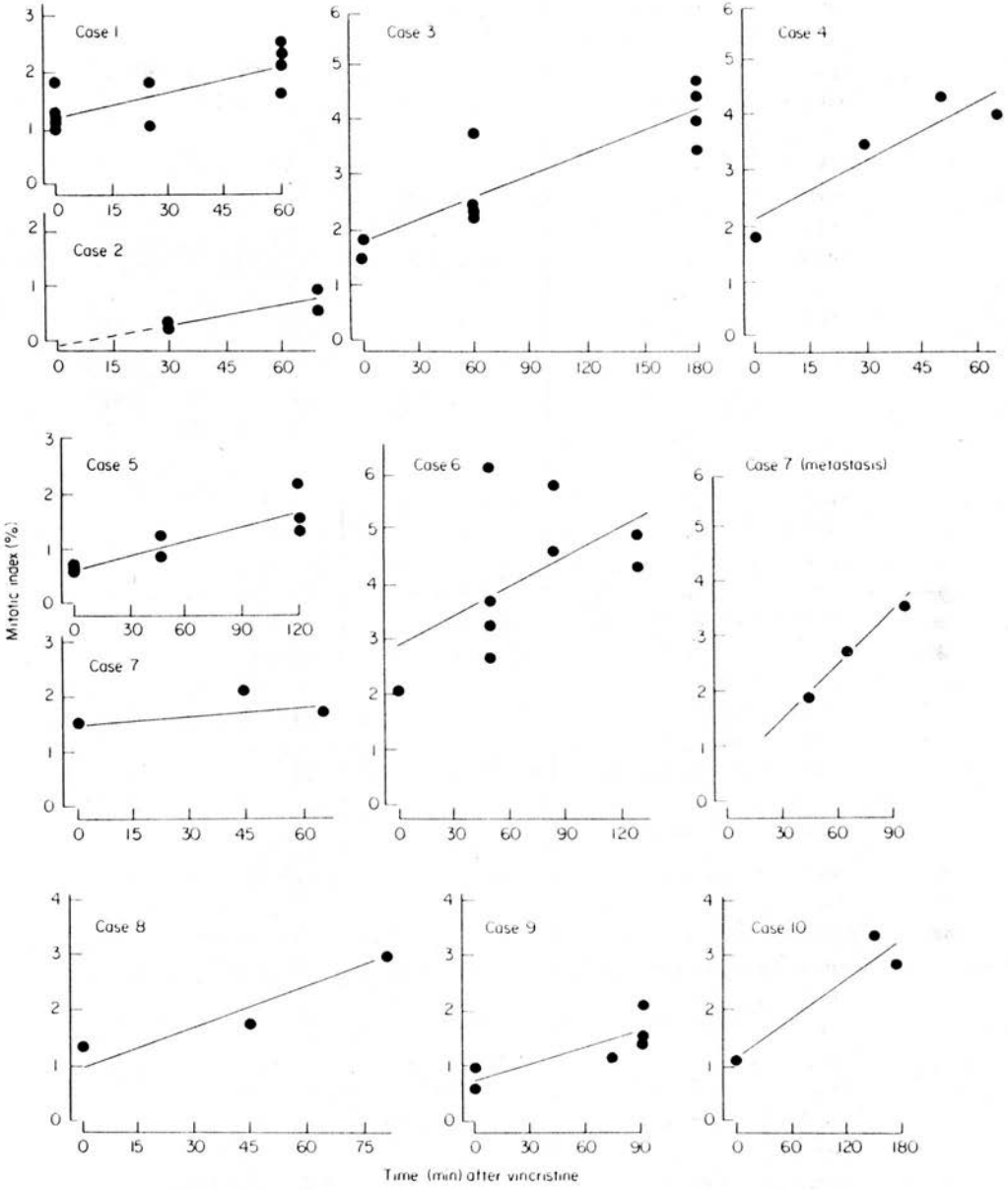

FIG. 1. Mitotic accumulation graphs for human gastric carcinoma after intravenous infusion of vincristine. The lines are fitted assuming a rectangular age distribution.

these tumours. In view of the confidence limits obtained, it would be invidious to compare the birth rates of these tumours one with another, or with the birth rates obtained for control mucosae, particularly in those cases with only two readings (e.g. case 11 and all control mucosa: see Tables 1 and 2).

TABLE 1. Kinetic parameters in gastric carcinoma (with 95% confidence limits)

Case No.	Cell production rate (cells/1000 cells/hr)	Potential doubling time (hr) (rectangular age distribution)	Potential doubling time (hr) (exponential age distribution)
1	8·6 (2·2, 15·1)	116 (66, 462)	82 (47, 325)
2	6·3 (0, 16·9)	157 (59, ∞)	109 (41, ∞)
3	8·1 (4·7, 11·5)	123 (87, 214)	88 (62, 153)
4	24·3 (4·1, 45·4)	41 (22, 242)	29 (16, 178)
5	4·9 (2·8, 6·9)	204 (144, 353)	143 (101, 247)
6	14·9 (1·3, 28·6)	67 (35, 766)	48 (25, 550)
7	3·4 (0, 58·8)	290 (17, ∞)	204 (12, ∞)
7 (metastasis 1)	20·8 (0, 52·6)	48 (19, ∞)	34 (13, ∞)
7 (metastasis 2)	27·4	36	
8	11·4 (0, 66·6)	88 (15, ∞)	62 (10, ∞)
9	5·5 (0·1, 10·2)	180 (98, 1072)	126 (69, 748)
10	7·6 (0, 27·8)	132 (36, ∞)	94 (25, ∞)
11	7·0	143	

Normal mucosae

In all instances where non-neoplastic tissues were analysed, only two post-vincristine biopsies were available. The preoperative endoscopic biopsies were inadequate, partly due to their small size, but mostly because of extreme difficulty in orientation. It is assumed that collection of metaphases was linear over the arrest period, and the birth rate is calculated from the difference between the readings. Confidence limits cannot be calculated in the usual manner, but from extrapolation from the tumour data where only three points were available (see case 10) it is clear that little information has been gathered.

The cell production rates for normal pyloric mucosa varied from 8 to 16 cells/1000 cells/hr, with a mean value of 12·7 cells/1000 cells/hr.

Abnormal mucosae

In one case, hyperplastic mucosae of both pyloric and acid-secreting types were available for analysis. These gave values of 30 and 22 cells/1000 cells/hr respectively. In two cases

TABLE 2. Cell production rates (CPR) and potential doubling time (t_{pd}) in human gastric mucosa

Case No.	Normal mucosa*		Hyperplastic mucosa		Intestinal metaplasia	
	CPR (cells/1000 cells/hr)	t_{pd} (hr)	CPR (cells/1000 cells/hr)	t_{pd} (hr)	CPR (cells/1000 cells/hr)	t_{pd} (hr)
3	8·2	122	30·5*	33*	21·5	46
			22·5†	44†		
4	16·0	62	—	—	—	—
5	15·1	66	—	—	—	—
6	12·6	79	—	—	33·0	30

* Pyloric type mucosa.
† Acid secreting mucosa.

intestinal metaplasia occurring in pyloric mucosa was analysed and gave values of 21 and 33 cells/1000 cells/hr respectively.

Hepatic metastases

In one case the liver contained several surface metastases; in one larger metastasis three biopsies were obtained, but in a smaller one only two were taken. Cell production rates of 20 and 27 cells/1000 cells/hr were found in these metastases, respectively. For the larger metastasis the confidence intervals are again large (Table 1).

DISCUSSION

The present results are unsatisfactory in indicating absolute values of proliferative rates, and also in any comparison of rates which might be made. The reason for the large confidence intervals lies in the few time periods which, for technical reasons, are available for study.

In the absence of information about variation between samples, little can be said with safety about the mean. A major limiting factor in these experiments is the number of biopsies which can be taken. Compared with previously published work (Refsum & Berdal, 1967; Fulker *et al.*, 1971; Camplejohn *et al.*, 1973) in terms of numbers of readings, the present results are considerably more detailed on an individual basis. Consequently, claims made for accurate individual t_{pd} values from these stathmokinetic studies are probably overstated. Of course, the variability between biopsies in the same case (so, for example, case 6) also contribute to the wide confidence limits.

There is certainly some difficulty in deciding upon an optimum metaphase arrest period; one must ensure that the period is not long enough to incur errors due to metaphase degeneration (Aherne & Camplejohn, 1972), but at the same time a short arrest period may increase sampling errors. Of practical importance is the duration of the operative procedure, which will limit the time period available for study. In this work, biopsies were obtained until the blood supply to the stomach was compromised.

Considering the normal pyloric mucosa, the mean cell production rate of 13 cells/1000 cells/hr does compare reasonably well with the value of 11 cells/1000 cells/hr which can be calculated from the data of Lipkin, Sherlock & Bell (1963). These workers calculated k_b for the proliferative region only, but their data can be converted to include all cells from the gland neck to the surface, which was the method used in the present study. It should, however, be remembered that the present confidence limits are likely to be large, and also that linearity of metaphase collection has been assumed. In this latter context, Wright *et al.* (1973) have shown in the human small intestine that calculations made from two points compare well with those made from multiple biopsies after vincristine.

As judged by mean values, cell production rates appear elevated in hyperplastic mucosae, and also in mucosa displaying intestinal metaplasia. This is particularly interesting, since intestinal metaplasia is held to be a premalignant lesion in the stomach, and the results are similar to those obtained by Croft (1972), who relied on mitotic counts for comparison.

It is obvious that the gastric blood supply will be compromised during the operation, and this could conceivably lead to an underestimate of the proliferative rate. Camplejohn *et al.* (1973) found that linearity of metaphase collection was not maintained in excision specimens, and these have not been used in this study. This would not exclude a systematic decrease in

mitotic indices; the apparent agreement of the present data with the ^{3}H-TdR results of Lipkin *et al.* (1963) suggest that proliferative rates are of the correct order, at least in the normal mucosae.

While it is reasonable to choose a rectangular or steady state age distribution for the normal and possibly the abnormal mucosae, the tumour presents a problem. At the time these studies were made, the tumours were considerably advanced. The decycling probability is probably large at this time, which would have the effect of lowering the proportion of young cells from that of an exponential age distribution: a high rate of cell loss at mitosis is also probable. These factors would combine to produce a near rectangular age distribution, and hence steady state equations are probably more appropriate.

Three cases were excluded because of inadequate metaphase arrest, which was readily detectable by the presence of anaphases and telophases in the late post-vincristine samples. There was no doubt that vincristine was given in these cases, and the observation may only be explained on the basis of different sensitivities of different tumour cells. It is just possible that, in these cases, the duration of mitosis is very long, with the result that no metaphases became arrested. However, in all the other cases studied, metaphase arrest was complete, with no evidence of anaphase escape, as early as 30 min after vincristine injection; consequently the mitotic duration in the cases included is relatively short.

It should perhaps be emphasized that, in the cases included, all biopsies taken after vincristine injection showed prophases and metaphases only, and there was no evidence of anaphase escape.

Besides the problems itemized above, there are several other sources of error in this technique. There is obviously a good deal of kinetic heterogeneity within a single tumour; Camplejohn (unpublished results) showed that considerable variability in mitotic indices existed between various areas in single neuroblastomas and rectal carcinomas. It is further assumed that vincristine acts *ab initio* and also that cells are cycling at the same rate, when in fact there is probably a distribution of transit times through the cell cycle. Finally it is assumed that linear regression analysis is the optimal method of fitting the data (Whittal, Appleton & Wright, 1975).

A further difficulty concerns the time of onset of metaphase action. In this study, in common with previous investigators, we have been obliged to take the native mitotic index as the first reading; in fact there may be a delay period before the onset of metaphase arrest, which would lead to a systematic underestimate of the cell production rate.

It would be easy to say that cell production is higher in hepatic metastases than in the primary tumour; also that because cell production rates are lower in tumours than in the adjacent normal mucosa, carcinoma of the stomach would be expected to be resistant to radiotherapy and phase-specific cytotoxic chemotherapy, which would be expected to kill more normal cells than tumour cells. This latter contention is supported by some results obtained by Hoffman & Post (1967) who demonstrated that labelling and mitotic indices were lower in a gastric carcinoma than adjacent mucosa.

However, it is not intended to give these data any more worth than they inherently possess by attempting to compare them with previous results, with the degree of differentiation of the tumour, or even with the normal mucosa, since the very wide confidence intervals do not allow accurate measurement or insight. Indeed, if this technique is to have any future, we must improve technical aspects in order to obtain many more biopsies during the course of the procedure.

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Reprinted from THE LANCET, *August* 7, 1971, *pp.* 279-283

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THYROID FUNCTION IN ACROMEGALY*

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Summary Thyroid-function studies were performed in 12 patients with active acromegaly. 1 patient had thyrotoxicosis, the rest were considered euthyroid although 3 of them had abnormal thyroid scans. All patients had a normal serum thyroid-stimulating-hormone (T.S.H.) level. The euthyroid group had renal and thyroid iodide clearances significantly greater than normal ($P < 0.05$ and $P < 0.01$ respectively). The absolute iodide uptake (A.I.U.) and 2½-hour radioiodine uptake by the thyroid were also significantly greater than normal ($P < 0.05$) and the plasma-inorganic-iodide (P.I.I.) was in the low normal range. The maximal binding capacity of thyroxine-binding prealbumin (T.B.P.A.) was increased and the maximal binding capacity of thyroxine-binding globulin (T.B.G.) was normal during the early years of active acromegaly. However, considering the duration of active disease (range 4–30 years) a significant linear diminution ($P < 0.05$) of both T.B.P.A. and T.B.G. could be demonstrated with time.

Introduction

THE function of the thyroid gland may be altered in acromegaly in several different ways. Secondary hypothyroidism can result from destruction of the thyrotroph cells in the pituitary, either by an enlarging pituitary adenoma or following therapeutic destruction of the gland by surgery or irradiation. Goitre is commonly associated with acromegaly; it was found in 24 out of 100 cases [1] and in 7 out of 27 cases.[2] Hyperthyroidism is uncommon, occurring in 3% of the above series, and primary hypothyroidism was not seen.

* Part of this article formed the basis of a paper read at the section of Endocrinology of the Royal Society of Medicine in May, 1971.

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Various alterations in thyroid-function tests have been described in association with acromegaly. Hamwi et al.[2] showed that in patients with active acromegaly the 24-hour radioiodine uptake was higher and the serum-protein-bound iodine was lower than in the inactive group. In contrast, McCullagh et al.[3] investigated patients with active acromegaly and hypermetabolism (mean basal metabolic rate +38%) and claimed that the 2-hour radioactive iodine uptake was low or low-normal (mean 20·75%, range 6–40%). However, their normal 2-hour radioiodine uptake was of the order of 16%, making this conclusion invalid.

In addition, various abnormalities of thyroid-hormone-binding proteins have been described. Inada and Sterling [4] found that the maximal binding capacity of thyroxine-binding prealbumin (T.B.P.A.) was normal or increased in patients with acromegaly of 5 years' duration or less, while the maximal binding capacity of thyroxine-binding α-globulin (T.B.G.) was diminished only after a decade or more of the disease.

The present study, which is part of a large survey of thyroid disease in acromegaly,[5] was undertaken to investigate in detail the thyroid function of a group of patients with active acromegaly, to ascertain whether any specific alterations of function were present.

Patients and Methods

12 patients with acromegaly, 8 women and 4 men, whose ages ranged from 21 to 63 years (mean 48 years) were investigated. 10 patients had not been treated previously for their acromegaly, but 1 patient had had pituitary surgery and another a course of deep-X-ray therapy. 1 patient who developed hyperthyroidism during the course of her acromegaly was included. She had a multinodular goitre and obvious clinical features of hyperthyroidism. All the other patients were considered euthyroid. In all 12 patients the acromegaly was considered active both clinically and biochemically. The clinical criteria were progressive acral growth, increased skin and heel-pad thickness, and excessive sweating. The main biochemical criterion was a raised serum-growth-hormone (G.H.) level on radioimmunoassay [6]—in particular, elevation of the fasting G.H. level, which was not suppressed below 5 ng. per 100 ml. at any time during a standard glucose-tolerance test.[7] The augmented insulin-tolerance test for detecting insulin resistance was also performed in most patients.[8]

An attempt was made to estimate the age of onset and probable duration of the disease. This was based on discussion with the patient and relatives about the various

symptoms and signs known to occur, and scrutiny of old photographs and hospital records. Although this was very difficult to achieve, it was believed that a reasonable assessment was made for each patient.

Two tests for thyroid antibodies were performed—the tanned red-cell (T.R.C.) test for thyroglobulin antibodies using formolinised cells (Burroughs Wellcome) (minimum positive titre 1/25) and the complement-fixation test (C.F.T.) for the detection of microsomal autoantibodies [9] (minimum positive titre 1/4). The serum-protein-bound-iodine (P.B.I.) was estimated by the 'Technicon' 'AutoAnalyzer' technique,[10] and the mean of at least two results was taken. 'Thyopac-3' test [11] was performed as an in-vitro indirect measure of serum-thyroid hormones based on protein-binding.[12] The free-thyroxine index was derived from the P.B.I. and thyopac-3 and compared with the normal range for euthyroid patients.[13] Serum-thyroid-stimulating hormone (T.S.H.) was measured using the double antibody radioimmunoassay technique of Hall et al.[14] The normal range was established from tests on 29 control subjects.

The radioiodine uptake of the thyroid gland was estimated at 1, 2½, 6, 24, and 48 hours after a tracer dose of 50 μC of ^{131}I given orally while fasting. All urine was collected between the 1-hour and 2½-hour period of the test, and the total and inorganic urinary iodides measured by the Technicon method.[10] The thyroid and renal iodide clearances were estimated, and the plasma inorganic-iodide (P.I.I.) and absolute-iodide uptake (A.I.U.) were calculated.[15] The normal ranges were those established in this unit during previous studies.[16] A thyroid scan ('Scintiscanning') was performed at the 24-hour point of the radioiodine-uptake test.

The maximal binding capacities for thyroxine binding to α-globulin (T.B.G.) and prealbumin (T.B.P.A.) were determined using the method employed by Launay.[17] ^{131}I-labelled L-thyroxine was obtained from the Radiochemical Centre, Amersham. The actual calculations by manual planimetry were those used by Oppenheimer et al.[18] 11 healthy volunteers (5 male and 6 female), aged 24–52 years, were used to give a normal range.

Results

Table I shows the clinical details of the 12 patients and results of the routine pituitary–thyroid function tests. Case 12 had hyperthyroidism. Thyroid enlargement, classified according to Kilpatrick et al.,[19] was found in 3 other cases. Only 1 patient (no. 8) had a positive thyroid-antibody test—T.R.C. test positive at a titre of 1/25, gland palpably soft but not visibly enlarged.

Considering the patients who were clinically euthyroid (nos. 1–11), the P.B.I. (median 6·5 μg. per

100 ml., range 5·4–7·5 μg. per 100 ml.) was within our normal range (median 6·6 μg. per 100 ml., range 3·0–8·8 μg. per 100 ml.). 10 of the 11 patients had thyopac-3 results within the normal range (0·91–1·22); patient no. 7 with a low (hyperthyroid) result—0·84—had a nodular goitre but was clinically euthyroid. The free-thyroxine index was within the normal range (3·7–8·6) in all 11 patients. All patients, including the patient with hyperthyroidism, had T.S.H. levels within the normal range (1·6 ± S.D. 0·8 microunits per ml.). The patient with clinical evidence of hyperthyroidism had a P.B.I., free-thyroxine index, and 6-hour radioiodine uptake within the hyperthyroid range.

The thyroid scans were abnormal in the 3 patients with stage-III thyroid enlargement, showing a patchy uptake consistent with a nodular goitre. One other patient (no. 1) had an abnormal scan with very little uptake demonstrated in the left lobe, although there was no clinical abnormality of the gland. Patient no. 8 with stage-I thyroid enlargement had a normal thyroid scan.

The activity of the disease in this group was confirmed biochemically by their raised fasting growth-hormone levels, which did not decrease normally during a standard 2-hour glucose tolerance test, and by the finding of insulin resistance in 7 of 10 patients, with an insulin-resistance index above the normal range (62–142).

10 of the 11 clinically euthyroid patients had their iodine metabolism results statistically analysed by computer for those modalities for which normal control values were available (table II). Patient no. 5 had a very high P.I.I. which seemed to have no physiological explanation, and this result, together with a high A.I.U., low thyroid clearance, and low radioiodine uptake, suggested excessive iodine intake. When this was discussed with the patient, she admitted that around the time of these tests she was taking an iodine-containing linctus for her bronchitis. Her results were therefore not included in the analysis. It was decided that a non-parametric test would be appropriate because of the skewness of the distributions involved. The Wilcoxon test for two samples was therefore used.[10]

Thyroid radioiodine uptake of the patient at 2½ hours (median 20·2%, range 12·0–27·8%) was significantly higher ($P < 0·05$) than in the normal group

TABLE I—CLINICAL DETAILS AND PITUITARY-THYROID FUNCTION TESTS

Case no.	Age (yr.)	Sex	Duration of disease (yr.)	Goitre	Thyroid scan	P.B.I. (μg./100 ml.)	Thyopac-3	Free thyroxine index	T.S.H. (μU./ml.)	Growth-hormone (ng./ml.)		Insulin resistance index
										Fasting	Lowest in G.T.T.	
1	41	F	9	0	Abnormal	7·0	1·12	6·3	0·6	9	28	199
2*	48	F	22	Stage 3 (nodular)	Abnormal	6·4	1·10	5·8	3·0	29	25	..
3	50	F	12–15	0	Normal	7·5	1·09	6·9	1·0	85	66	148
4	55	F	12	0	Normal	5·4	0·98	5·5	..	11·5	7	80
5	61	F	30	0	Normal	7·2	1·15	6·3	0·9	7	5·5	122
6	63	F	17+	0	Normal	6·6	1·15	5·7	1·5	96	90	150
7†	63	F	15+	Stage 3 (nodular)	Abnormal	7·2	0·84	8·6	<0·5	85	78	172
8	21	M	9	Stage 1	Normal	6·1	1·01	6·0	0·9	59	53	162
9	39	M	16	0	Normal	6·5	0·92	7·1	<0·5	52	36	..
10	41	M	14	0	Normal	6·4	1·11	5·8	1·7	120	112	201
11	50	M	2–5	0	Normal	6·4	0·98	6·5	1·0	88	70	85
12	45	F	4	Stage 3 (nodular)	Abnormal	13·4‡	1·05	12·8	1·0	380	510	193

* Decompression optic chiasm at age 28 years. † Deep-X-ray therapy to pituitary at age 60 years. ‡ Radioiodine uptake at 6 hours = 58%.

TABLE II—IODINE METABOLISM STUDIES

	Radioiodine uptake (% dose) at:					Thyroid clearance (ml./min.)	Plasma-inorganic-iodide (μg./100 ml.)	Absolute iodide uptake (μg./hr.)	Renal iodide clearance (ml./min.)
	1 hr.	2½ hr.	6 hr.	24 hr.	48 hr.				
Case no.									
1	..	25·7	35·1	54·7	..	..	0·12	1·2	48·5
2	7·6	16·3	25·7	38·3	40·0	25·4	0·11	1·7	46·3
3	6·2	20·2	37·2	54·2	54·6	42·0	0·20	5·0	20·4
4	7·6	..	..	30·2	..	..	0·26	..	62·3
5*	4·9	7·4	12·3	22·2	24·1	8·9	1·93	10·3	36·5
6	8·1	13·2	20·3	32·6	33·7	29·8	0·17	3·0	35·8
7	14·2	27·8	39·5	56·5	53·5	62·9	0·13	4·7	43·9
8	7·6	12·0	18·0	31·3	33·0	30·8	..	..	110·0
9	11·8	23·1	34·0	50·0	51·0	66·1	0·13	5·2	59·4
10	11·9	23·0	28·2	38·3	36·9	35·2	0·19	3·9	17·0
11	10·1	16·7	27·0	44·4	..	43·1	0·15	3·8	60·5
Median ..	8·1	20·2	28·2	41·3	40·0	38·6	0·15	3·8	47·4
Normal median	8·5	15·0	..	37·3	..	17·0	0·19	1·8	29·0
Range	1·2–12·1	3·9–19·6	..	25·5–45·7	..	9·0–30·9	0·13–0·29	1·0–4·3	21·8–37·3
Significance ..	N.S.	$P < 0·05$	..	N.S.	..	$P < 0·01$	N.S.	$P < 0·05$	$P < 0·05$

* On iodine-containing linctus in study period; results therefore omitted from calculations.

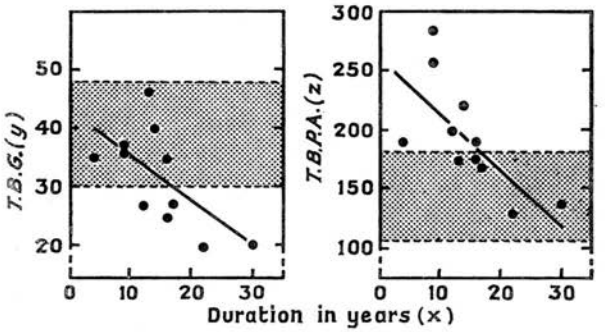
(median 15·0%, range 3·9–19·6%). Thyroid radioiodine uptake values of the patients at 1 and 24 hours were not significantly different from normal. At 1 hour the patients' median uptake was 8·1% (range 6·2–14·2%), the normal median uptake being 8·5% (range 1·2–12·1%). At 24 hours the patients' median uptake was 41·3% (range 30·2–56·5%), and the normal median uptake was 37·3% (range 25·5–45·7%).

The thyroid clearance of radioiodine of the patients (median 38·6 ml. per minute, range 25·4–66·1) was significantly higher ($P < 0.01$) than in the normal group (median 17·0 ml. per minute, range 9·0–30·9). Plasma-inorganic-iodide (P.I.I.) levels of the patients (median 0·15 μg. per 100 ml., range 0·11–0·26) were lower than those of the normal group (median 0·19 μg. per 100 ml., range 0·15–0·29), but just failed to achieve the conventional 5% significance level. Absolute iodide uptake (A.I.U.) values in the patients (median 3·8 μg. per hour, range 1·2–5·2) were significantly higher ($P < 0.05$) than those of the normal group (median 1·8 μg. per hour, range 1·0–4·3). Renal iodide clearance of the patients (median 47·4 ml. per minute, range 17·0–110) was significantly higher ($P < 0.05$) than in the normal group (median 29·0 ml. per minute, range 21·8–37·3).

The results of the maximal binding capacity of T.B.G. and T.B.P.A. estimations, together with the age, sex, and estimated duration of disease for the 11 euthyroid patients, are shown in table III. In the patients the range for T.B.G. capacity was 20–46 μg. per 100 ml. and for T.B.P.A. 130–285 μg. per 100 ml. Since there seemed to be a definite relationship between T.B.G. and

TABLE III—MAXIMAL BINDING CAPACITY OF THYROXINE-BINDING PREALBUMIN (T.B.P.A.) AND OF THYROXINE-BINDING ALPHA-GLOBULIN (T.B.G.)

Case no.	Age (yr.)	Sex	T.B.P.A. (μg./100 ml.)	T.B.G. (μg./100 ml.)	Duration of disease (yr.)
1	41	F	258	37	9
2	48	F	130	20	22
3	50	F	175	46	12–15
4	55	F	200	27	12
5	61	F	139	20	30
6	63	F	170	27	17+
7	63	F	190	35	15+
8	21	M	285	36	9
9	39	M	175	25	16
10	41	M	220	40	14
11	50	M	192	35	2–5



Maximal binding capacities of T.B.G. and T.B.P.A. related to duration of disease for 11 patients.

Shaded areas = normal ranges (mean ± 2 S.D.).
$Y = 43{\cdot}36 - 0{\cdot}796X$; $r^2 = 0{\cdot}44$.
$Z = 262{\cdot}40 - 4{\cdot}644X$; $r^2 = 0{\cdot}49$.

T.B.P.A. capacities and duration of disease, graphs were plotted as between these modalities (see accompanying figure). The normal range (mean ± 2 S.D.) is indicated; for T.B.G. capacity it is 29·6–48·2 μg. per 100 ml. and for T.B.P.A. capacity it is 103·9–184·9 μg. per 100 ml. Comparing T.B.G. and T.B.P.A. capacities in the patients with the normal range, in both instances the gradient of the line with time was significantly different from zero at the 5% significance level. The gradient showed a decrease of both T.B.G. and T.B.P.A. capacities over the period 4–30 years—the T.B.G. capacity being initially within the normal range and the T.B.P.A. capacity above the normal range.

Discussion

While it is generally accepted that the incidence of goitre is increased in acromegaly, critical comparisons of the frequency of goitre in acromegalics with that in the general population of the particular area of study are lacking. The sample examined in this study is too small for accurate assessment of goitre prevalence, though there seems little doubt that the finding of 4 unequivocally abnormal thyroid scans in 12 subjects selected only on the criterion of active acromegaly supports a thyroid abnormality in this disease.

The present studies of thyroid function provide information as to the various factors which might play a part in the thyroid abnormality in acromegaly. Previous suggestions [3] that goitre and hyperthyroidism resulted from coincidental T.S.H. overproduction by

the eosinophil tumour are disproved, since in all the euthyroid subjects with or without goitre, and in the hyperthyroid patient, serum T.S.H. levels were normal, as they were in the patients studied by Raud and Odell.[21] Similarly, Hall et al.[14] have demonstrated normal T.S.H. levels in patients with non-toxic goitre, if subjects with autoimmune thyroid disease, dyshormonogenetic goitre, and previous destructive therapy to the thyroid are excluded.

At present there is only one apparently well-documented case of chromophobe adenoma in which both acromegaly and hyperthyroidism have been found simultaneously with an increased secretion of T.S.H.[22] Critical analysis of the data provided does not necessarily support the conclusion of these workers, since T.S.H. levels were first measured during the period that the patient was receiving carbimazole and iodides (about 66 mg. of iodide daily). It has recently been shown that this drug combination rapidly raises T.S.H. in normal subjects,[14] and this patient, as well as having a nodular goitre, had evidence of autoimmune thyroid disease, which is known to sensitise the thyroid to the action of iodides.[23,24] A week after thyroidectomy the T.S.H. titre of the patient was unaltered, as might be expected from persistence of antithyroid drug action. Triiodothyronine (T_3) in doses of 100 μg. per day for 7 days did not cause any reduction in T.S.H. level, though the McKenzie bioassay is not sufficiently sensitive to reveal minor changes in T.S.H. and Murray and Stewart [25] have shown that, in some patients treated with iodides, more prolonged administration of T_3 is required to cause full suppression of thyroid activity. In conclusion, the evidence presented does not necessarily justify the claim that T.S.H. overproduction can cause hyperthyroidism in patients with acromegaly. A case has been reported of a chromophobe adenoma associated with hyperthyroidism due to increased T.S.H. production, demonstrated by radioimmunoassay, but the patient did not have acromegaly.[26]

The only hyperthyroid patient had a long-standing nodular goitre and had recently developed clinical and biochemical features of hyperthyroidism. The finding of thyroid scans typical of nodular goitre in 3 other subjects also suggests that the abnormality leading to thyroid overactivity is likely to arise on the basis of a nodular goitre where one or more nodules becomes

autonomous and hyperfunctional. Factors known to be involved in the development of hyperthyroidism in patients with thyroid nodules include the mass of the nodular tissue and the iodine supply in the diet.[27]

Growth hormone itself is likely to be responsible for the generalised visceromegaly in acromegaly, and the thyroid is probably included in this process. Short-term administration of G.H. has no physiologically significant effect on thyroxine homœostasis in man,[28] but a sustained increase in growth-hormone production has been suggested to lead to goitre formation because of the increased needs for thyroid hormone.[29] Though the mechanism of this action is not clear, any latent thyroid defect, such as an enzyme defect [29] or perhaps subclinical autoimmune thyroid disease,[30] might be expected to be unmasked in acromegaly.

Secondary factors which might lead to goitre include relative iodine deficiency and abnormalities of thyroid-hormone-binding proteins. The significantly increased renal-iodide clearance which has been demonstrated in acromegaly is likely to be related to the increased renal size [1] and is certainly one factor which might lead to the lowered P.I.I. Increased renal-iodide clearance has been shown to lower the P.I.I. in some cases of simple goitre [31] and in pregnancy.[32] A secondary sequel of iodine deficiency—increased thyroid iodide clearance—is seen in all iodine deficiency states [15]; but in mild iodine deficiency, unlike acromegaly, the A.I.U. is normal. In severe iodine deficiency, as seen in endemic goitre, compensation fails and the A.I.U. is low and T.S.H. levels are raised. The significantly raised A.I.U. in acromegaly may be related to an increased functioning thyroid mass—as supported by the significantly increased radioiodine uptake at 2½ hours—in association with only minor iodide deficiency. These changes of thyroid function were not reflected by abnormalities in serum-protein-bound iodine or thyopac-3 levels.

The results of the maximal binding capacities of T.B.P.A. and T.B.G. agree with those of Inada and Sterling [4] and Hollander et al.[33] in showing high T.B.P.A. capacities in recent-onset acromegaly and a diminution of T.B.G. capacities in long-standing cases. The present results indicate that initially the T.B.P.A. capacity is high and that of T.B.G. is normal. As the

duration of the disease increases, there is a gradual but progressive reduction in both T.B.P.A. and T.B.G. capacities, and not, as has been suggested for the T.B.P.A. capacity, merely a return to the normal range. The effects of age [34] and sex [35] on the maximal binding capacities seemed to have no bearing on the results obtained.

It is interesting to speculate on possible mechanisms for the abnormalities of thyroid-hormone-binding proteins. Oppenheimer et al.[36] have shown that T.B.P.A. capacity is closely related to serum-albumin concentration, and Ulrich et al.[37] have demonstrated an increased rate of albumin synthesis in hypophysectomised rats in response to G.H. It is likely, therefore, that the raised level of T.B.P.A. results from a direct action of G.H. on synthesis of the protein; maximal binding capacity of T.B.P.A. is known to be directly related to its serum concentration.[18,38] Raised levels of binding protein will provide a further stress on the thyroid by causing a temporary reduction in free-thyroxine, which must be compensated by increased hormone release. It has been suggested that the eventual diminished capacities of T.B.P.A. and T.B.G. may be due to the long-standing hypermetabolic state and prolonged catabolic effects, since a similar reduction of binding proteins occurs in active thyrotoxicosis.[39]

In conclusion, it appears that the excessive growth-hormone production in acromegaly is associated with, and probably responsible for, a variety of thyroidal and extrathyroidal abnormalities, some of which may play a part in the pathogenesis of the nodular goitre of acromegaly.

The advice of Prof. G. A. Smart, Dr. F. Clark, and Prof. F. Farmer is gratefully acknowledged, as is the technical assistance of Mrs. E. Burleigh, Mrs. A. Hewison, and Mrs. J. Amos. This work was supported by grants from the Wellcome Trust. Human T.S.H. for iodination was supplied by the National Pituitary Agency; standard T.S.H. was supplied by Dr. M. Cotes, of the Medical Research Council; and T.S.H. for antiserum production was a gift of Dr. A. Hartree.

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Printed in Great Britain

The Lancet Office, 7, Adam Street, Adelphi, London, W.C.2.

David Appleton

Clinical Endocrinology (1980) **12,** 71–79

EPIDEMIOLOGY OF ACROMEGALY IN THE NEWCASTLE REGION

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(Received 25 May 1979; revised 12 July 1979; accepted 24 July 1979)

SUMMARY

In an attempt to derive estimates of the incidence and prevalence of acromegaly a survey of the population in the area served by the former Newcastle Regional Hospital Board has been conducted to detect cases of acromegaly alive after 1 January 1960 and diagnosed before 31 December 1971. Cases were detected by means of letters to general practitioners, hospital physicians, neurosurgeons and to hospital records officers, as well as from death certificates. From the population of 3·1 millions a firm diagnosis of acromegaly was made in 164 patients, in eighty-one on clinical grounds alone and in eighty-three with confirmation by assay of human growth hormone (hGH). The annual incidence of acromegaly appears to be close to three cases per million and the prevalence of diagnosed cases up to forty cases per million. In male acromegalics there was a significant increase in the risk of death from cardiovascular, cerebrovascular, respiratory and malignant diseases but in females from cerebrovascular disease only.

The syndrome of acromegaly, first described by Marie (1886), is easily recognizable and its clinical features, presentation and complications are well documented. However, the prevalence and natural history of the disorder are not accurately known since previous work has been based on selected groups of patients usually attending specialist units (Gordon *et al.*, 1962; Hamwi *et al.*, 1960; Kanis *et al.*, 1974) although specialist bias has been reduced in one study (Wright *et al.*, 1970) by including patients from five separate hospitals. Unfortunately, any hospital series is likely to be selected since mild cases may not be referred because they are thought not to require treatment and long standing advanced cases are often considered to be inactive and may not be followed in hospital clinics.

In the present study an attempt has been made to trace all patients with acromegaly in the area administered by the former Newcastle Regional Hospital Board. The survey has

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been limited to those alive on 1 January 1960 and in whom the diagnosis of acromegaly was made before 31 December 1971. It is appreciated that detection is likely to have been incomplete, but we consider that the stability of the population in this region relative to other areas of Britain minimizes the problems of finding patients.

PATIENTS AND METHODS

The survey began in Newcastle upon Tyne in 1967 and was extended to the whole region in 1968 when letters were sent to all general practitioners on the Executive Council lists, to hospital consultants and to records officers, in order to trace patients with acromegaly who were alive, or who had died after 1 January 1960. Copies of all death certificates for the region on which acromegaly or a pituitary tumour was mentioned were obtained from the Registrar General and, after scrutiny, further details were sought in cases where there seemed a possibility of acromegaly. The search was continued until 31 December 1971, so that in all a 12 year period was covered; it was hoped that this would provide information on the incidence as well as the prevalence of acromegaly.

Where possible patients attended hospital at least once for clinical and biochemical assessment. In those patients available for study but unable to attend hospital, assessment, including physical examination and an oral glucose tolerance test (GTT, obtaining basal and half-hourly specimens of blood for 2 h for estimation of human growth hormone (hGH) and glucose), was performed at home. Following assessment treatment was offered if indicated and further management supervised from the hospital.

The criteria adopted for the diagnosis of acromegaly were a history of a period of progressive acral growth in adult life, supported when possible by photographic evidence of altered features, and radiological evidence of increased skin and heel pad thickness. Radiological evidence of enlargement of the pituitary fossa provided valuable confirmatory evidence of the diagnosis particularly in patients assessed before the availability of hGH assay. Precise biochemical diagnosis became possible only with the development of the radioimmunoassay of hGH (Hartog *et al.*, 1964). In normal subjects serum hGH is suppressed below 5 mi.u./l (1mi.u. = 1 μg of Medical Research hGH Standard A which we used as a standard in the assay) during a standard oral glucose tolerance test (Hall *et al.*, 1974). Failure of suppression below this level was taken as biochemical confirmation of active acromegaly.

The age of onset of the disease was estimated by careful questioning of the patient and relatives, and by scrutiny of old photographs and hospital records. This procedure, we believe, resulted in a reasonable assessment in the majority of cases. When such an estimate was not possible, usually in patients who could not be interviewed, no attempt was made to guess the duration of the disease.

In order to calculate the incidence and prevalence of the disease and details relating to mortality, we recorded the patients age, sex, estimated duration of disease, year of onset, duration to diagnosis, age at death and cause of death, together with relevant clinical and biochemical features. In addition, any treatment, together with resultant changes, and related morbidity and mortality were noted and will be reported separately.

The population of the region in 1968 was obtained from the Regional Hospital Board. Mortality in the region was assumed to be similar to that of the rest of the country and use was made of the Registrar General's Statistical Review of England and Wales for the year

1969. The spatial distribution of cases was considered in relation to population density and distance of the patient's home from Newcastle (the investigating centre).

RESULTS

Methods by which patients traced

Of the 164 patients analysed, fifty-four had been referred to the Newcastle Endocrine Unit for investigation, twenty-three were notified as a result of the circular and the remaining eighty-seven patients were traced through hospital record departments and death certificates.

Basis of diagnosis

One hundred and sixty-four patients fulfilled the criteria for inclusion in the survey. In eighty-one the diagnosis of acromegaly was based solely on clinical grounds with typical facies and extremities, serial photographic changes and usually enlargement of the pituitary fossa. In the remaining eighty-three patients the clinical diagnosis was confirmed by failure of suppression of hGH during a GTT. Thirty-five patients in whom the diagnosis of acromegaly was considered possible were excluded from analysis either because hGH suppressed normally or, in the absence of hGH measurements, there was insufficient other evidence of acromegaly.

Clinical features

Fifty-eight of the 164 patients presented because of a change in their features and fifty-six because of associated disturbances, especially visual field defects, carpal tunnel syndrome and headaches. The remaining fifty patients had no complaints directly attributable to acromegaly but were noted to have acromegalic features when seeking medical advice for an unrelated complaint. The clinical severity, judged by the enlargement and alterations of the extremities and face, was mild in twelve patients, moderate but constant in forty-four, moderate and worsening in sixty-six, gross but constant in fifteen and gross and progressing in twelve. The remaining fifteen patients could not be classified in this way because of insufficient data.

Growth hormone levels

In eighty-three patients hGH was measured during GTT before any treatment was given. Minimum hGH during this suppression test was 5–10 mi.u./l in seven (8%), 11–30 mi.u./l in twenty-five (30%), 31–100 mi.u./l in thirty-six (43%) and greater than 100 mi.u./l in fifteen (18%).

Geographical distribution of patients

The distribution of detected cases of acromegaly differed significantly over the region (Table 1). The greatest numbers were found in areas closest to the Endocrine Unit in Newcastle.

Duration of disease

The estimated year of onset was considered to be sufficiently accurate in 127 patients (Fig. 1) to allow assessment of the duration till diagnosis (Fig. 2); this ranged from 1 to 33 years. The mean duration till diagnosis was 6·6 years, but, because of the skewness of the

Table 1. The prevalence of acromegaly in different parts of the Newcastle region

District	Number of patients with acromegaly	Total population	Acromegaly per 100 000
Northumberland	20	430 860	4·6
Newcastle	36	398 960	9·0
Gateshead	12	171 860	7·0
Durham county	52	925 230	5·6
Teesside	31	851 240	3·6
Cumberland	13	314 050	4·1
Total	164	3 092 200	5·3

The variation in these prevalence rates is greater than would arise by chance alone, $\chi^2 = 17{\cdot}09$ with 5 d.f., $P < 0{\cdot}01$.

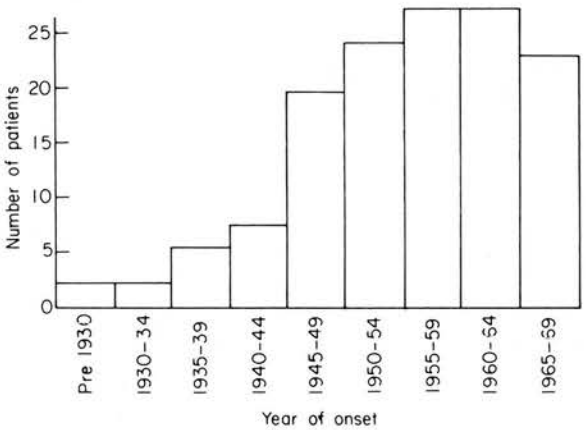

Fig. 1. The year of onset of acromegaly determined by questioning the patients and relatives and by scrutiny of old photographs (127 patients).

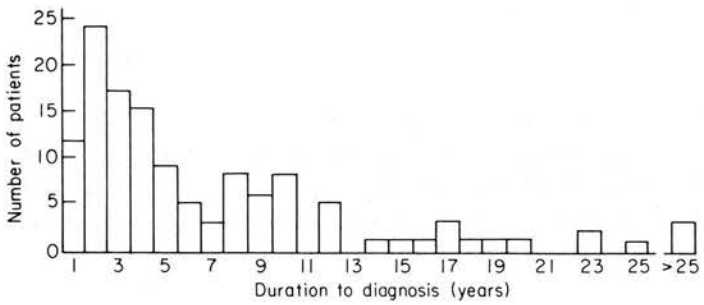

Fig. 2. The duration to diagnosis derived from the year of diagnosis (Fig. 3) and the year of onset (Fig. 1). The mean was 6·6 years and the median 3·7 years.

distributions involved it is more appropriate to use the median: 50% of cases had been diagnosed by 3·7 years. The year of diagnosis is shown in Fig. 3; the survey began in 1967 and this probably accounts for the increased rate of diagnosis in the years 1967–1970, and for the large number of patients diagnosed within 4 years of development of the disease. (Fig. 1).

The duration of acromegaly till death was known in twenty-two of the forty-five patients dying during the 12 years covered by the survey (Fig. 4). This shows that very few patients survive more than 30 years from onset, the mean duration till death being 15·4 years (Median 14·0 years).

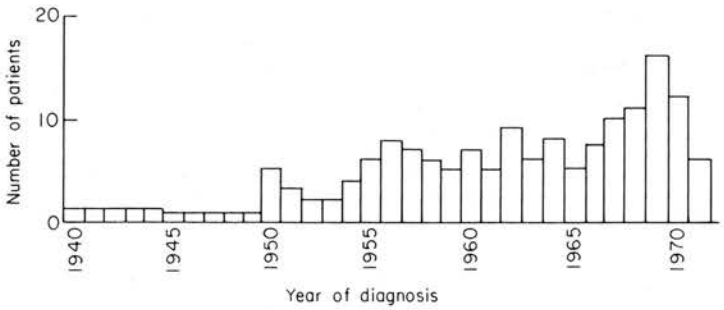

Fig. 3. The year of diagnosis data shows an increase in the years 1967–70 which is probably due to increased interest generated by the survey and to the availability of the radioimmunoassay for growth hormone.

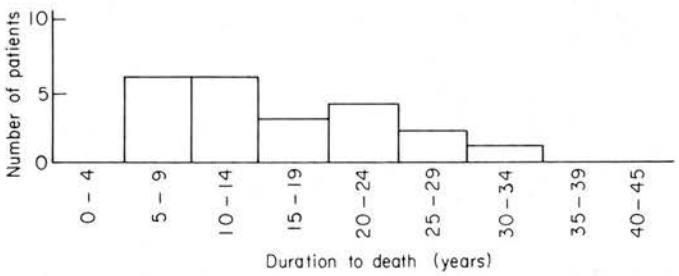

Fig. 4. The duration of the disease to death was known in only twenty-two of the forty-five patients dying during the survey. These may not be representative of all those dying since the duration to death is not greater than the duration of disease in those still surviving. The mean was 15·4 years and the median 14·0 years.

Age distribution of living patients and age at death

The age distribution of patients alive at the end of the study is shown in Fig. 5. It can be seen that a peak is reached in the 50–54 age group. Examination of the figures for the age at death (Fig. 6) shows them to be consistent with the fall in the numbers of living cases from the age of 55.

Death rates

From data on the year of diagnosis and, where relevant, the year of death, it was

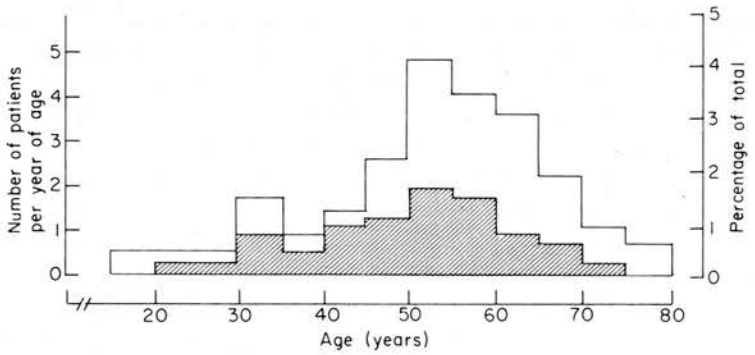

Fig. 5. Age of 119 patients alive on 1 January 1972. The mean age of patients alive at the end of the study was 52·5 years with most patients in the 50–54 age group. The pattern was similar in males (▨) and females (□). The median was 54 years.

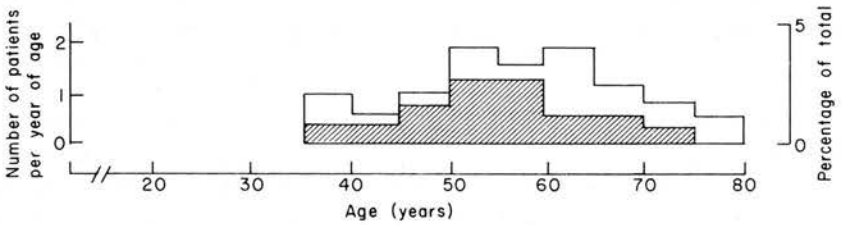

Fig. 6. Age at death of forty-five patients. Most deaths occurred over the age of 50, mean age 56·8. This increased death rate accounts for the falling numbers of survivors above the age of 55 (Fig. 5). The pattern was again similar in males (▨) and females (□). The median was 57 years.

Table 2. Death rate in acromegalics compared with those expected in the population

	Male (70)		Female (94)	
Cause	Expected	Observed	Expected	Observed
Malignancy	1·3	6†	2·3	1
Respiratory	0·8	5†	1·0	2
Cardiovascular	1·9	8‡	2·6	5
Cerebro-vascular	0·4	4‡	1·3	10‡
Total	5·0	24‡	8·6	21‡

The disease categories used correspond to those in the Registrar General's Review as follows: malignancy, 140–239; respiratory, 460–519; cardiovascular, 393–429; cerebro-vascular, 430–438.

† $P<0{\cdot}01$.

‡ $P<0{\cdot}001$.

determined that the seventy males in the survey were at risk for a mean of 5·3 years and the ninety-four females for a mean of 7·2 years. Assuming that the male and female age distributions did not change throughout the study and were as shown in Fig. 5, it is possible to calculate the number of deaths expected from various causes in a group of seventy males and ninety-four females with the same age structure as our patients but subject to the death rates for England and Wales as a whole (Registrar General's Statistical Review of England and Wales for 1969, Table 17a). On the further assumption that the number of deaths is Poisson-distributed it is possible to compare the observed number with the expected number (Table 2). In males total deaths and those in each category investigated are significantly higher than those in the general population; for the female group the total deaths and those from cerebrovascular disease are significantly increased.

DISCUSSION

The lower prevalence of acromegaly in areas distant from Newcastle may be due to a less complete response to our enquiries in these areas rather than a failure to diagnose the condition or a true reduction in prevalence of the disease. If the Newcastle prevalence of nine cases per 10^5 population were representative of the whole region, approximately 270 patients (rather than 164) should have been traced during the survey.

It is possible to make several estimates of the incidence of acromegaly in the population: since there were 103 cases diagnosed in the 12 years from 1960–1971 and the population remained reasonably steady at 3·1 millions, a mean annual incidence of 2·8 per million may be derived. This could be an overestimate if the rate of diagnosis increased during the years of the survey, 1967–71, but is likely still to be low for the reason given in the previous paragraph. The forty-five deaths which occurred during the 12 years of the survey suggest an annual incidence of 1·3 cases per million, but this is certainly an underestimate because some patients dying in the early years covered by the survey are bound to have been missed, and in addition it is conceivable that with improved management in recent years death may be delayed, so that incidence could, at least temporarily, exceed death rate.

Perhaps the best method of estimating the incidence is to combine the information relating to duration till diagnosis, duration till death, and year of onset. From the distribution of durations till diagnosis (Fig. 2) we can calculate that about 33% of those for whom the year of onset was between 1965 and 1969 will not have been diagnosed by the end of the survey; likewise 12% from 1960–64, 7% from 1955–59 and 5% from 1950–54 will not have been covered by the survey. From the distribution of durations till death (Fig. 4) we can similarly deduce that about 27% of those for whom the year of diagnosis was between 1950 and 1954 will have died before the start of the period covered by the survey; 54% from 1945–49, 68% from 1940–44, 86% from 1935–39, 95% from 1930–34, and virtually all patients whose year of onset was before 1930 will be missing from the survey. This gives a distribution of the year of onset for those studied as shown in Fig. 7; the value for the annual incidence which makes this most like Fig. 1 is 2·6 cases per million if we take the period prevalence of 164 cases to be accurate, and 4·2 cases per million if we believe that the Newcastle figure for prevalence should be applied to the whole region.

From these various approaches it would appear that the incidence of acromegaly in the population of 3·1 millions studied is around ten per annum. By considering the distribution of durations till diagnosis again we can show that at any one time a number of

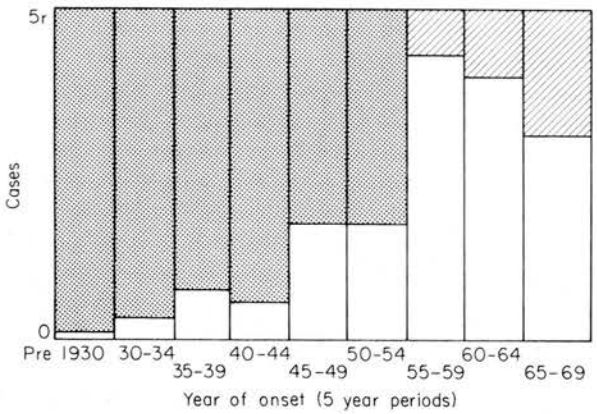

Fig. 7. A model to show the predicted pattern of year of onset of acromegaly diagnosed at 31 December 1971 derived from data on duration to death and to diagnosis (see text). The value for the annual incidence which makes this correspond with the observed pattern of year of onset (Fig. 1) is 4·9% of the total, i.e. 2·6 or 4·2 per million depending on whether the Newcastle or the regional figures for period prevalence are used. (▨) Undiagnosed, (▤) died before 1960, (□) alive at beginning of survey. r = Annual incidence of cases ultimately diagnosed.

acromegalics equal to about five and a half times the annual incidence remain to be diagnosed; 62% of those will have had the disease for less than 5 years and 26% between 5 and 10 years. The point prevalence of diagnosed cases at the end of the survey was 119, or about thirty-eight per million population.

The finding of a preponderance of female cases, though a barely significant one, is in agreement with other large series (Gordon *et al.*, 1962; Wright *et al.*, 1970). The mean age of those living and the mean age at death were also similar to expected results (Wright *et al.*, 1970) but it was of interest that only fifty-eight of the 164 cases presented complaining about their appearances, which is in contrast to the findings of other groups (Lawrence *et al.*, 1970; Kanis *et al.*, 1974) and is probably largely explained because patients in these studies were being referred for possible treatment.

Mortality and associated diseases

There was a significantly higher mortality in both males and females than for the population as a whole and in males an increased number of deaths occurred from all causes examined, namely, malignancy, respiratory, cardiovascular and cerebrovascular disease. In females, only deaths from cerebrovascular disease significantly exceeded the number expected. The overall mortality rate in our patients was similar to that reported by Wright *et al.* (1970) but there were differences in the frequency of the various causes of death. The overall mortality rate in the treated cases (22%), was better than in the untreated group (40%), but the reasons for this are complex and will be discussed more fully elsewhere.

ACKNOWLEDGEMENTS

We wish to thank Mrs Mary Turnbull for her valuable assistance in helping with the hospital notes necessary for the preparation of this paper and Mrs M. E. Shepherd for typing the manuscript.

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THYROTROPHIN RESPONSE TO THYROTROPHIN-RELEASING HORMONE IN OPHTHALMIC GRAVES' DISEASE: CORRELATION WITH OTHER ASPECTS OF THYROID FUNCTION, THYROID SUPPRESSIBILITY AND ACTIVITY OF EYE SIGNS

69

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(*Accepted for publication* 27 *June* 1973)

SUMMARY

Thirty-four patients with ophthalmic Graves' disease were investigated by routine thyroid function tests, by measurement of the serum thyroid-stimulating hormone (TSH) response to thyrotrophin-releasing hormone (TRH) and by measurement of the serum triiodothyronine (T_3) levels.

The patients could be divided into four groups according to their response to TRH—normal, impaired, absent and exaggerated. Those with normal responses had routine thyroid function tests and serum T_3 levels which in general did not differ from normal control values. Those with impaired and with absent responses showed routine thyroid function tests which approached the hyperthyroid range together with significant elevation of serum T_3 levels. It is suggested that they might represent examples of 'subclinical T_3 thyrotoxicosis'. Patients with exaggerated responses had routine thyroid function tests near the lower end of the normal range and could be regarded as suffering from 'subclinical hypothyroidism'. There was a good correlation between a normal TRH response and normal thyroid suppressibility by T_3, and between impaired and absent responses and impaired thyroid suppressibility. It is evident that the TRH test which is safer, shorter and more convenient, can replace the T_3 suppression test in routine clinical practice. The response to TRH also provided information on the activity of the eye signs, a normal response was associated with improving eye signs whereas impaired, absent or exaggerated responses were associated with static signs or actual deterioration. The TRH test is of value in the diagnosis of unilateral exophthalmos since about three-quarters of the patients in this series showed some abnormality. The wide range of TRH response and of circulating thyroid hormone levels is to be

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expected since the thyroid is not subject to the normal finely-balanced negative feed-back mechanism via TSH.

INTRODUCTION

Ophthalmic Graves' disease is the term used to describe patients exhibiting the ocular manifestations of Graves' disease in the absence of hyperthyroidism or a past history of hyperthyroidism (Rundle & Wilson, 1945; Hall *et al.*, 1970). Most patients have normal routine thyroid function tests but in a minority there is clinical and/or biochemical evidence of thyroid failure. Some thyroid abnormality can be detected by clinical examination or by laboratory tests in the majority of patients (Hall *et al.*, 1970; Werner, 1955). Measurement of the serum thyroid-stimulating hormone (TSH) response to thyrotrophin-releasing hormone (TRH) has provided a sensitive method of detecting minor degrees of thyroid dysfunction (Fleischer *et al.*, 1970; Ormston *et al.*, 1971; Hall *et al.*, 1972). Patients with primary thyroid failure show an exaggerated and prolonged TSH response to TRH whereas those with hyperthyroidism fail to respond. It has been suggested that an absent TSH response to TRH correlates with lack of suppressibility of the thyroidal radioiodine uptake by triiodothyronine (T_3) (Lawton *et al.*, 1971; Ormston, 1972), one of the common abnormalities of thyroid function in ophthalmic Graves' disease (Werner, 1955; Hobbs, 1963; Bayliss, 1967; Bowden & Rose, 1969). We have therefore investigated the TSH response to TRH and its relationship to thyroid suppressibility and other tests of thyroid function including direct measurement of the serum triiodothyronine levels in patients with ophthalmic Graves' disease.

PATIENTS AND METHODS

Thirty-four patients with ophthalmic Graves' disease were studied. All were clinically euthyroid at the time as assessed by two independent observers. Uniformity of assessment was ensured by the use of standard indices of thyroid function (Gurney *et al.*, 1970; Bilewicz *et al.*, 1969). The patients were asked whether their eyes were static, improving or deteriorating.

The TRH test was performed as previously described (Ormston *et al.*, 1971), measuring serum TSH levels before, and 20 and 60 min after the intravenous injection of 200 μg of synthetic TRH. Serum TSH was measured by a double antibody radioimmunoassay (Hall *et al.*, 1971), using Medical Research Council 68/38 human TSH as standard. The response to TRH was classified into four groups (Hall *et al.*, 1972) as: normal (a rise in serum TSH in men of 4·2 to 16·1 μU/ml and in women 5·9 to 18·2 μU/ml); impaired (a rise of between 1 and 4·2 μU/ml in men and from 1 to 5·9 μU/ml in women); absent (a rise of less than 1 μU/ml); and exaggerated (a rise greater than 16·1 μU/ml in men and 18·2 μU/ml in women).

Routine thyroid function tests employed included the serum protein-bound-iodine (PBI, Technicon 'AutoAnalyzer' method 56), the Sephadex uptake of ^{125}I-labelled triiodothyronine ('Thyopac-3') (Clark & Brown, 1970a), and by calculation from these two measurements, the free-thyroxine-index (FT_4 index) (Clark & Brown, 1970b).

Serum T_3 levels were measured by radioimmunoassay on unextracted serum by the method of Hesch & Evered (1973). The maximal binding capacities for thyroxine (T_4) to thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA) were deter-

TABLE 1. Thyroid function tests and activity of eyes in patients with ophthalmic Graves' disease

Patients	PBI (μg%) Mean ± SD (1)	Thyopac-3 ratio to control Mean ± SD (2)	FT_4 index Mean ± SD (3)	T_3 (ng/ml) Mean ± SD (4)	Thyroid 6 hr ^{131}I uptake (%) Before T_3 Mean ± SD (5)	Thyroid 6 hr ^{131}I uptake (%) After T_3 Mean ± SD	TRC no. (6)	CFT no. (7)	Activity of eyes Improving	Activity of eyes Static	Activity of eyes Deteriorating
Normal TSH response to TRH (9)	5·70 ±0·95	1·07 ±0·04	5·31 ±0·75	1·23 ±0·44	20·5 ±3·7	6·4 ±3·7	5	5	8	1	0
Impaired TSH response to TRH (7)	7·57 ±1·52	1·11 ±0·08	6·80 ±1·10	2·04 ±0·23	22·6 ±8·5	13·6 ±3·2	2	2	3	4	0
Absent TSH response to TRH (10)	6·82 ±1·23	1·06 ±0·04	6·45 ±1·27	1·75 ±0·25	30·8 ±10·5	25·8 ±8·8	2	4	1	3	6
Exaggerated TSH response to TRH (8)	4·33 ±1·11	1·13 ±0·06	3·84 ±0·99	0·85 ±0·31	22·9 ±8·2 24·8*	9·5 ±7·2 6·3*	5	8	2	2	4
Control series	5·38 ±1·43 (77)	1·06 ±0·08 (135)	6·17 ±1·24 (108)	1·26 ±0·23 (54)	25·6 ± 7·3 (72)	—	0·5%	5%	—	—	—

(1) Normal range 2·5–8·2 μg/100 ml. (2) Normal range 0·91–1·22. (3) Normal range 3·7–8·6. (4) Normal range 0·79–1·73 ng/ml, mean 1·26. (5) Normal range 11·0–40·2%. (6) Titre >1/25 = positive. (7) Titre >1/8 = positive.

* Excluding two patients with detectable LATS.

mined by the method of Launay (1966) as previously described (Mukhtar *et al.*, 1971). Triiodothyronine (T_3) suppression tests were performed by measuring the 6 hr thyroidal uptake of radioiodine before and 7 days after the daily administration of 120 μg of T_3.

Antibodies to thyroid and gastric cytoplasm were determined by immunofluorescence, thyroglobulin antibodies by tanned red cell haemagglutination, and thyroid cytoplasmic antibodies by complement fixation as described in the World Health Organisation Manual for Autoimmune Serology. Long-acting thyroid stimulator (LATS) was measured by a modification of the McKenzie bioassay (Clark *et al.*, 1973).

RESULTS

Clinical features

The eye signs of the thirty-four patients (twenty-five women and nine men) were as follows:

Exophthalmos	8 unilateral	13 bilateral
Lid retraction	10 unilateral	3 bilateral
Ophthalmoplegia	6 unilateral	7 bilateral
Swollen eyelids	10 unilateral	18 bilateral

Goitres were present in 12 patients

Thyroid function tests

Table 1 shows the results of thyroid function tests in control subjects and in the thirty-four patients with ophthalmic Graves' disease grouped according to their TSH response to TRH. It also shows a subjective assessment by the patients of the activity of their eye signs.

Table 2 shows the levels of significance (given by *t*-tests) for the differences in the thyroid

TABLE 2. Comparison of thyroid function tests in patients with ophthalmic Graves' disease grouped according to their response to TRH and in control subjects

Groups compared	Significance (*P* value)			T_3	^{131}I (% drop)
	PBI	Thyopac-3	FT_4 index		
Control *v.* Normal	NS*	NS	0·05	NS	—
Impaired	0·001	NS	NS	0·001	—
Absent	0·01	NS	NS	0·001	—
Exaggerated	0·05	0·05	0·001	0·001	—
Normal *v.* Impaired	0·01	NS	0·01	0·001	0·001
Absent	0·05	NS	0·05	0·01	0·001
Exaggerated	0·05	NS	0·01	NS	NS
Impaired *v.* Absent	NS	NS	NS	0·05	0·05
Exaggerated	0·001	NS	0·001	0·001	NS
Absent *v.* Exaggeraged	0·001	0·05	0·001	0·001	NS

* Not significant.

function tests in the four groups of patients and the controls. Patients with a normal TRH response differed from control subjects, having a lower free thyroxine index and a lower 6 hr thyroidal radioiodine uptake. The explanation for these differences is not clear, they

might reflect the presence of significant autoimmune thyroid disease in the patients with ophthalmic Graves' disease but this seems unlikely in view of their normal basal TSH levels and responses to TRH. Those patients with exaggerated TRH responses had significantly lower PBI, 'Thyopac-3', FT_4 index and T_3 values than controls. In the groups with impaired or absent response the serum PBI and T_3 were significantly higher than in the normal response group and normal controls. The percentage fall in the thyroidal radioiodine uptake after T_3 in the impaired and absent response groups was significantly less than in the normal response group. Comparison of the impaired and absent response groups shows that the former had a significantly higher serum T_3 level yet a greater suppression of the thyroidal radioiodine uptake by T_3.

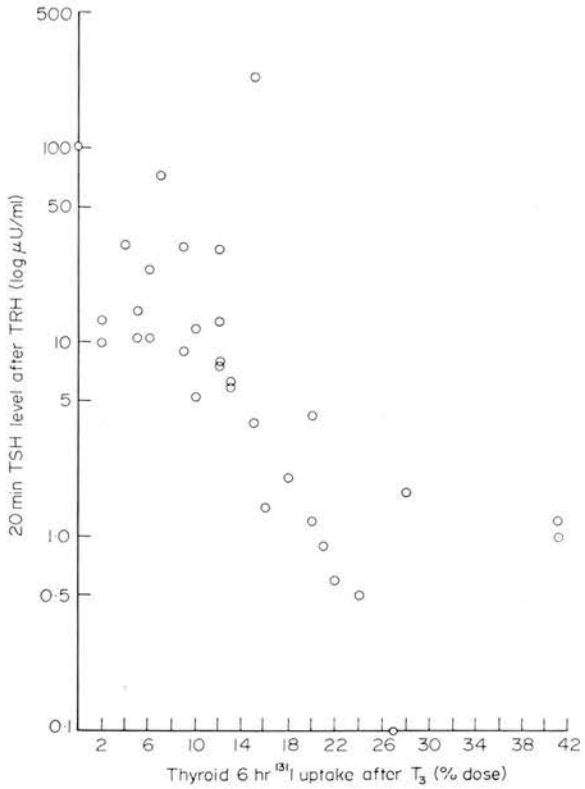

FIG. 1. Relationship between 20 min TSH value after TRH and thyroidal 6 hr ^{131}I uptake after T_3 in patients with ophthalmic Graves' disease (correlation coefficient −0·64).

Fig. 1 shows the highly significant relationship between the 20 min TSH value after TRH and the thyroidal 6 hr ^{131}I uptake after T_3 in the thirty-four patients. A similar relationship existed between the 60 min TSH level after TRH and the thyroidal radioiodine uptake (correlation coefficient −0·64).

Patients with a normal TSH response to TRH had a significantly greater frequency of improving eye signs ($P<0·01$) compared with those with impaired, absent or exaggerated responses where the eye signs were mainly either static or deteriorating.

TBG and TBPA capacities in the patients with ophthalmic Graves' disease were within the wide normal ranges previously described. Thyroid antibodies were found much more frequently than in control subjects and in higher titres than in hyperthyroid patients with Graves' disease, the frequency and titre of antibodies being similar to those seen in patients with Hashimoto's thyroiditis.

DISCUSSION

The expression 'ophthalmic type of Graves' disease' was first used by Rundle & Wilson (1945) to describe patients in whom the ocular changes of Graves' disease were present in the absence of a goitre or hyperthyroidism. The definition has since been modified to include patients with a goitre providing they are not hyperthyroid on clinical examination and by routine thyroid function tests and give no past history suggestive of hyperthyroidism (Hall *et al.*, 1970). Patients with clinical and/or biochemical evidence of hypothyroidism can also be included in this grouping since ophthalmopathy characteristic of Graves' disease has been described in patients with histologically-proven Hashimoto's disease (Wyse *et al.*, 1968).

A variety of abnormalities of thyroid function can be found in patients with ophthalmic Graves' disease, including lack of suppressibility of the thyroidal radioiodine uptake by T_3, a greater than normal suppression of thyroid uptake by iodides, impaired thyroid reserve demonstrated by TSH stimulation and an increased frequency of thyroid and gastric autoantibodies. Limitation of thyroid reserve is only found in a minority of patients and is not therefore a valid explanation of the euthyroid state in ophthalmic Graves' disease.

Clinical examination and routine thyroid function tests are relatively crude methods of assessing minor degrees of thyroid dysfunction. By using the thyrotrophin-releasing hormone test (Ormston *et al.*, 1971) and the radioimmunoassay of serum T_3 (Hesch & Evered, 1973) we have been able to determine whether the previously described abnormalities of thyroid function are associated with any alteration in thyroid hormone secretion. A normal TSH response to TRH reflects normal circulating levels of T_4 and T_3 and it has been shown that the range of T_4 and T_3 in which the TSH response to TRH is normal is very small (Snyder & Utiger, 1972). This observation is confirmed in patients with ophthalmic Graves' disease where those with a normal TRH response showed circulating thyroid hormone levels which were close to the mean for normal subjects. The finding of impaired or absent responses to TRH in half the patients in this series suggested that they should have an increased circulating thyroid hormone level. This suggestion was confirmed by measurement of the serum PBI, although the values did not exceed the upper limit of the conventional normal range. However, direct measurement of the serum T_3 in patients with absent or impaired responses to TRH showed mean values considerably above those seen in normal subjects, despite the absence of symptomatic hyperthyroidism. It therefore seems likely that the raised levels of circulating thyroid hormones seen in some patients with ophthalmic Graves' disease are responsible for their absolute or relative unresponsiveness to TRH.

A small group of patients showed an exaggerated and prolonged TSH response to TRH similar to that seen in hypothyroidism. Their routine thyroid function tests were significantly lower than those of patients with a normal response yet most remained within the normal range. However, the serum T_3 level was below the lower limit of normal in most patients. All of the patients in this group had high titres of complement-fixing thyroid antibodies

indicative of significant autoimmune thyroiditis and could be regarded as examples of subclinical hypothyroidism (Evered & Hall, 1972).

The wide range of TSH response to TRH in patients with ophthalmic Graves' disease mirrors the wide range of their thyroid hormone secretion. It would appear that the TSH response to TRH is largely modulated by the serum T_3 concentration in this clinical situation. It is evident that elevation of the serum T_3 level is not necessarily associated with clinical hyperthyroidism. Hollander *et al.* (1971) have suggested that many patients with classical thyrotoxicosis go through an initial phase of 'T_3 thyrotoxicosis'. This explanation may be valid in certain series of patients with ophthalmic Graves' disease, where the majority have eventually developed overt hyperthyroidism (Hobbs, 1963). However, it is not valid in our series of patients since most had been under observation for several years without developing hyperthyroidism. They might therefore be regarded as showing a relatively stable state of 'subclinical T_3 thyrotoxicosis'.

There was a good correlation between the results of the TRH test and the T_3 suppression test. Patients with a normal TRH response almost invariably showed normal suppressibility whereas those with absent or impaired responses usually failed to suppress normally. This confirms previous reports (Lawton *et al.*, 1971; Ormston, 1972), based on small groups of patients, that an absent TSH response to TRH correlates with lack of thyroid suppressibility, but is not necessarily an indication of hyperthyroidism. Two patients with exaggerated responses failed to suppress with T_3 and in both significant levels of LATS were detected. Failure of suppression of the thyroidal radioiodine uptake by T_3 implies that the thyroid trapping process is controlled by a mechanism other than TSH, e.g. some intrathyroidal autoregulatory process, LATS, circulating lymphocytes, or a combination of these factors. It is for this reason (i.e. lack of normal feed-back control through TSH) that a wide range of levels of circulating thyroid hormone could be expected.

In clinical practice the TRH test can replace the T_3 suppression test since it provides at least as much information yet is safe, rapid and does not require the administration of a radioisotope or a potentially toxic drug to the patient. Like the T_3 suppression test, the TRH test is of value in the diagnosis of unilateral exophthalmos, 74% of the patients in this series showed some abnormality of the TRH response whereas only 59% showed impaired T_3 suppressibility. The TRH test is also of value in the prognosis of the eye signs, a normal response is usually associated with improving eye signs whereas impaired, absent and exaggerated responses are more often associated with static eye signs or deterioration.

To conclude, measurements of serum T_3 levels and of the TSH response to TRH have demonstrated a wide range of thyroid function in patients with ophthalmic Graves' disease ranging from subclinical hypothyroidism to subclinical hyperthyroidism. It appears that thyroid function in these patients represents a continuum with that observed in classical Graves' disease with hyperthyroidism. TRH responsiveness has been shown to correlate with thyroid suppressibility and to provide useful information on the activity of the eye signs.

ACKNOWLEDGMENTS

The advice of Dr Joyce Nutt, Dr M. Duffus, Dr H. Holgate and Dr J. Kilborn and the technical assistance of Mrs A. Hewison and Mrs M. McKendrick is gratefully acknowledged. This work was supported by grants from the Wellcome Trust, the Medical Research Council and the Newcastle University Hospital Group. M.R.C. Standard 68/38 TSH was supplied

by Dr M. Cotes, human TSH for preparation of antibodies by Dr A. Hartree and TSH for iodination by the National Pituitary Agency. Routine thyroid function tests were performed in the Department of Clinical Biochemistry under the direction of Professor A. L. Latner and Dr P. Smith whose help is gratefully acknowledged. TRH was kindly provided by Roche Products Ltd.

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Assessment of thyroid hormone assays

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J. clin. Path., 1976, **29**, 1054-1059

Assessment of thyroid hormone assays

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SYNOPSIS Four techniques for estimating serum T4 and three for estimating serum T3 have been investigated and found to be satisfactory in routine use. Normal ranges for each technique have been established. Estimation of serum T3 by the commercial kits tested appears to have a high discriminant value in the diagnosis of hyperthyroidism, although the diagnostic definition used inevitably enhances the apparent sensitivity of these techniques. Estimation of serum T4 will identify the majority of patients with symptomatic hypothyroidism. The low sensitivity of T3 in the diagnosis of thyroid failure is confirmed.

There has been a dramatic increase in the number of diagnostic techniques available for the investigation of suspected thyroid disease over the last two decades. The relative merits and limitations of the procedures currently available have been extensively reviewed (Evered, 1974; Evered *et al*, 1976) and a strategy for the investigation of the three common thyroid problems, that is, suspected hyperthyroidism, suspected hypothyroidism, and goitre, has been developed by these and other workers (Britton *et al*, 1975). The increasing use of new *in vitro* tests of thyroid function, the proliferation of commercially available kits, and problems of definition and classification have all led to confusion in the minds of many physicians and biochemists. The objective of this paper is to investigate the reliability and discriminant value of *in vitro* techniques for measuring total serum thyroxine (T4) and total serum triiodothyronine (T3) in the investigation of thyroid disease.

Definitions

HYPERTHYROIDISM

Hyperthyroidism was said to be present in subjects with clinical features of thyrotoxicosis and a raised serum triiodothyronine concentration by an established assay (Hesch and Evered, 1973). It is accepted that this definition is open to question, but it has the merit of specificity although it may be disputed on the grounds of sensitivity.

HYPOTHYROIDISM

Hypothyroidism was said to be present in subjects with clinical features of hypothyroidism and a raised serum thyrotrophin (thyroid stimulating hormone; TSH) concentration (Evered *et al*, 1973).

Asymptomatic (or subclinical) forms of the disease were excluded by definition.

Received for publication 10 May 1976

Materials

NORMAL SUBJECTS

Eighty normal subjects studied using all the techniques investigated were derived from laboratory and other hospital staff. Patients with non-thyroidal illness were not studied in view of the known effect of illness upon circulating thyroid hormone levels (Carter *et al*, 1974). The normal ranges for T4 using the Thyopac-4(R) kit and for T3 using the technique of Hesch and Evered (1973) and the Radiochemical Centre T3 kit were also established on a randomly selected sample of a non-hospital population. Full details of this study—the Whickham Community Survey—have been published elsewhere (Tunbridge *et al*, 1975).

SUBJECTS WITH THYROID DISEASE

Subjects with thyroid disease were seen consecutively in the Newcastle Endocrine Clinic after referral for assessment by one of three consultant endocrinologists (Professor R. Hall, Dr F. Clark or Dr D. C. Evered). Forty hyperthyroid and 40 hypothyroid subjects were studied.

PREGNANT SUBJECTS

Twenty pregnant subjects were drawn from a local antenatal clinic (Mr T. Lind) or were subjects who were pregnant when seen in the Whickham Com-

munity Survey. All were in the second or third trimester of pregnancy.

SUBJECTS ON AN ORAL CONTRACEPTIVE

One hundred and eighteen subjects receiving an oral contraceptive were drawn from the Whickham Community Survey.

Methods

SERUM THYROXINE ESTIMATION

Competitive protein-binding assays (CPBA)

CPBA was carried out using the Thyopac-4(R) kit from the Radiochemical Centre, Amersham. A small comparative study was carried out using a column technique as described by Seligson and Seligson (1972).

Radioimmunoassay (RIA)

Serum T4 was measured by RIA using two techniques:

(a) Newcastle assay: Specific T4 antisera were raised by the serial injection of T4 methyl ester hydrochloride conjugated with bovine serum albumin into New Zealand White rabbits. Standard T4 solutions were prepared by the technique of Murphy (1965). Hormone free serum was prepared by charcoal extraction. The assay technique is as follows:

0·475 ml buffer (with or without standard T4)
Barbital buffer—50 mM pH 8·6
0·025 ml unknown or hormone free serum
0·1 ml ANS (400 μg)
0·1 ml ^{125}I T4 (500 pg/tube)
0·1 ml antiserum
Incubate overnight at 4°C.
Separate with methyl-cellulose/charcoal.
Aspirate supernatant after 10 minutes.

(b) Commercial assay: T4 RIA was also carried out using a commercially available kit supplied by the Radiochemical Centre, Amersham.

SERUM TRIIODOTHYRONINE ESTIMATION

Serum T3 was measured by RIA using three techniques.

Newcastle assay

T3 RIA was measured using a modification of the technique described by Hesch and Evered (1973). The only differences from the original assay lay in the use of barbital buffer (50 mM pH 8·6) and ANS as a blocking agent.

Commercial assays

T3 RIA was carried out using the kits which are marketed by the Radiochemical Centre, Amersham and Hoechst Pharmaceuticals.

THYROID HORMONE BINDING TEST (THBT)

THBT was performed using a ^{125}I-T3 Sephadex uptake (Thyopac-3, Radiochemical Centre, Amersham).

TSH

Serum TSH was measured by a double-antibody RIA (Hall *et al*, 1971).

Analysis of data

The data derived from these studies were analysed using standard statistical techniques. The distribution of thyroid hormone concentrations within the Community is not significantly skewed, and thus it was possible to calculate means and standard deviations without transformation of the data. The sensitivity and specificity of each technique was assessed. Sensitivity is assessed by the number of affected subjects detected in relation to the number affected in the population tested. Specificity is evaluated as 1 minus the number of false positives as a proportion of the total number of non-affected subjects.

Thus, sensitivity is calculated:

$$\text{Sensitivity } (\%) = \frac{a}{a + c} \times 100$$

and specificity as:

$$\text{Specificity } (\%) = \frac{d}{b + d} \times 100$$

Where a = diseased subjects abnormal by the test (true positives)
b = non-diseased subjects abnormal by the test (false positives)
c = diseased subjects normal by the test (false negatives)
d = non-diseased subjects normal by the test (true negatives)
(Holland and Whitehead, 1974)

Results

TECHNICAL FACTORS

All the techniques gave acceptable degrees of precision and reproducibility. The intra-assay coefficient of variation ranged between 3·4 and 6·3% and interassay coefficient of variation between 5·0 and 9·0% for T4 and T3 assays (table I). The Thyopac-3 was shown to be highly precise and reproducible.

Technique	*Precision (within assay variation: coefficient of variation)*	*Reproducibility (between assay variation: coefficient of variation)*
T4		
Thyopac-4	4·2	5·0
CPBA—column	6·3	9·0
RIA—Newcastle	6·0	6·9
RIA—Amersham	4·6	7·7
T3		
RIA—Newcastle	5·5	8·7
RIA—Amersham	3·4	8·1
RIA—Hoechst	6·3	—
THBT		
Thyopac-3	0·7	1·9

Table I *Precision and reproducibility (per cent)*

	n	*Mean*	*SD*	*Percentiles* 2·5	97·5	
Thyroxine						
CPBA (Thyopac-4)	80	99·00	32	57	147·00	
	1685	105·00	22	63	153·00	WS[1]
CPBA (Column)	20	95·00	26	52	148·00	
RIA (NC)	80	108·00	23	67	149·00	
RIA (RCC)	80	103·00	19	64	141·00	
Triiodothyronine						
RIA (NC)	80	1·75	0·51	0·95	2·70	
	255	1·66	0·54	0·92	2·61	WS[1]
RIA (RCC)	80	2·67	0·55	1·77	3·77	
	255	2·69	0·60	1·84	4·15	WS[1]
RIA (Hoechst)	80	1·77	0·41	1·08	2·38	

Table II *Normal values (nM/l)*

Normal ranges derived from 80 hospital personnel. [1]Additional data for T4-CPBA (Thyopac-4) and T3—RIA (NC) and RIA (RCC) derived from the Whickham Survey (WS).

NORMAL SUBJECTS

The mean values obtained for T4 ranged between 95 and 108 nM/l. The limits of normality were assumed to be between the 2·5 and 97·5 percentile points for the population studied (table II). These ranges match closely those established by using 2SD above and below the mean. The values obtained are in close agreement with those derived from 1685 normal subjects in the Whickham Survey.

There was good agreement between the mean values for T3 using the Newcastle and Hoechst assays, although a narrower range was observed with the latter. The Newcastle values were closely matched by those obtained in the Whickham Survey population. The RCC assay consistently measured mean values 0·9-1·0 nM/l higher than the other two assays. The values obtained in the study of 80 laboratory personnel were closely matched by values obtained from a random subsample of 255 subjects from the survey population assayed over a six-month period (table II).

PREGNANCY AND OESTROGEN PREPARATIONS

Serum T4 and T3 values were significantly raised in subjects taking an oral contraceptive (n = 118) and during pregnancy (n = 20) (table III). Calculation of a free thyroxine or a free triiodothyronine index corrected the majority (96%) of values to within the previously established ranges for normality.

	Mean	*SD*	*Observed range*
T4			
CPBA (Thyopac-4)	121	31	71-252
FT_4T	96	26	51-174
RIA (NC)	162	30	123-226
FT_4T	117	21	93-163
T3			
RIA (NC)	2·24	0·64	1·12-4·15
FT_3T	1·85	0·55	0·95-3·04
RIA (RCC)	3·36	0·71	2·07-5·53
FT_3T	2·68	0·60	1·76-4·56
RIA (Hoechst)	2·00	0·32	1·24-2·38
FT_3T	1·43	0·22	0·83-1·81

Table III *Pregnancy and oestrogen preparations (all values in nM/l)*

SUBJECTS WITH THYROID DISEASE

Hyperthyroidism

The range of serum T4 and T3 values observed in hyperthyroidism using the techniques listed is shown in table IV. Estimation of the serum T4 discriminated well between euthyroid and hyperthyroid subjects when this variable was measured by RIA. The CPBA (Thyopac-4), however, proved to be a poor discriminant. A small comparative study using a column technique for the estimation of T4 yielded values comparable to those obtained by RIA. It was clear (see figure) that the Thyopac-4 technique progressively underestimated T4 values greater than 125 nM/l. Estimation of serum T3, however, by all techniques discriminated absolutely between euthyroid and hyperthyroid subjects in the present study (table V).

	Hyperthyroidism (40)	*Hypothyroidism (40)*
Thyroxine		
CPBA (Thyopac-4)	114->260	<10-63
RIA (NC)	150-420	<10-67
RIA (RCC)	129->300	<10-75
Triiodothyronine		
RIA (NC)	2·76-> 7·5	<0·15-1·72
RIA (RCC)	4·45->15·0	<0·15-3·02
RIA (Hoechst)	2·76-> 9·0	<0·15-1·90

Table IV *T4 and T3 values (nM/l) in thyroid disease*

Hypothyroidism

The range of serum T4 and T3 values seen in hypothyroidism is shown in table IV. Serum T4 levels were low in approximately 90% of subjects using all the techniques studied in patients with symptomatic

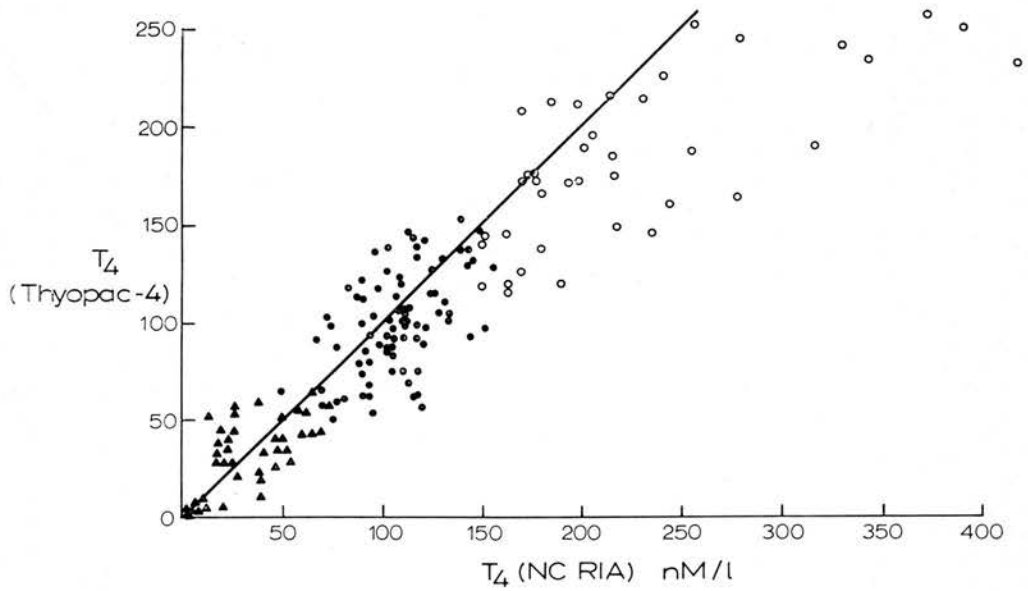

Figure *Serum T4 measured by RIA (NC) and CPBA (Thyopac-4) in 80 normal, 40 hypothyroid, and 40 hyperthyroid subjects. Line at 45°.*

	Sensitivity				*Specificity*
	Hyperthyroidism		*Hypothyroidism*		
	>97·5 percentile	*>2SD above mean*	*<2·5 percentile*	*>2SD below mean*	
T4					
CPBA (Thyopac-4)	65	65	88	91	93
RIA (NC)	100	100	97	97	96
RIA (RCC)	95	95	89	89	94
T3					
RIA (NC)	100 (by definition)	100	56	45	99
RIA (RCC)	100	100	54	51	98
RIA (Hoechst)	100	100	55	47	96

Table V *Sensitivity and specificity (per cent)*

hypothyroidism. The sensitivity of these techniques was not improved by calculating the free thyroxine index. Estimation of serum T3 proved to be an insensitive index of hypothyroidism. The sensitivity of these techniques was not materially increased by calculating a free triiodothyronine index (table V).

The specificity of all the techniques tested was high (93-99%) by comparison with the methods used for definition.

Discussion

The development of supraregional and regional radioimmunoassay services has placed hormone assays within the reach of every physician, and the availability of hormone assay kits has made it possible for radioimmunoassay techniques to be performed in nearly all hospital laboratories. No significant technical problems were encountered with any of the commercial kits used, and the precision and reproducibility were acceptable with all the assay techniques used.

The selection of an appropriate normal (reference) population poses many problems and no universally acceptable criteria have been established. The traditional use of blood donors, hospital personnel or the relatives of patients has many practical advantages, but the use of these groups does involve processes of selection which yield subjects who are frequently unrepresentative of the general population

in terms of age, sex, and socioeconomic group distribution. The normal ranges in this study have been established on a group of hospital personnel, but these results appear to be applicable to the general population since the ranges established using three of the techniques matched closely those seen in the Whickham Survey (Tunbridge *et al*, 1975). The sample seen in this survey was a 1 in 6 random sample taken from the electoral role in Whickham, County Durham. The response rate in the survey was 82·4%, and the sample closely matched the whole population of that community and the UK, in age, sex, and socioeconomic group structure. The range for normality was established on those subjects without any marker of thyroid disease or on any medication which would alter thyroid function tests (that is, subjects with thyroid antibodies, a raised TSH, a goitre, a personal or family history of thyroid disease, and those taking an oral contraceptive). There was good agreement between assays on the range of T4 in the reference population (60-150 nM/l). There was, however, one important and major difference in T3 values measured. The RCC T3 kit consistently measured higher values than the other two assays used which gave comparable results. The pack leaflet supplied with this kit acknowledges this difference and also indicates that quite markedly different values were obtained from reference populations in different centres. The explanation for these differences is not apparent, but it seems improbable that they can be attributed to regional variations in the light of the results of other assays of T3, but it is possible that they may represent differences in the selection of normal subjects. It is well established that non-thyroidal illness or cold surgery can lead to a significant reduction in measured T3 levels (Carter *et al*, 1974; Burr *et al*, 1975). The importance of each laboratory establishing its own normal range and appropriate care in the selection of a reference population (Holland and Whitehead, 1974) cannot be stressed too highly.

The expected increase in T4 and T3 levels was seen in pregnant subjects and those taking an oral contraceptive. The calculation of a free thyroxine or triiodothyronine index corrected the majority of values into the normal range. This calculation will not fully correct the thyroid hormone values in those subjects with the highest thyroxine binding globulin (TBG) capacities.

Estimation of the serum T4 by radioimmunoassay proved an effective discriminant of hyperthyroidism in the majority of cases. Estimation using the Thyopac-4 kit was, however, less satisfactory, and if this technique is used (together with a T3 assay) the prevalence of T3 thyrotoxicosis will be overestimated. This assay progressively underestimated the T4 value by comparison with the column technique and radioimmunoassay at T4 concentrations greater than 125 nM/l. The major difference between this technique and the column method lies in the extraction procedure, and it seems probable that this step accounts for the differences. This underestimation of higher T4 values has been reported before using a CPBA technique with an extraction procedure. The estimation of T3 appears to be a highly sensitive index of hyperthyroidism, and this view is consistent with earlier reports (Britton *et al*, 1975; Shalet *et al*, 1975). It must, however, be admitted that the diagnostic definition used inevitably enhances the apparent sensitivity of these techniques. It is unfortunate that there is no other acceptable, independent, and sensitive laboratory criterion against which these techniques can be assessed—as with TSH in the diagnosis of primary hypothyroidism. T3 may also be raised in some clinically euthyroid subjects and these groups have been reviewed elsewhere (Evered, 1975). It is possible that hyperthyroidism could exist with a normal T3 in a patient with a reduced TBG capacity or in one with a serious illness depressing the T3 concentration. Calculation of a free triiodothyronine index should correct the T3 value in the former situation and a thyrotrophin releasing hormone (TRH) test would be required to confirm the diagnosis in the latter case (Evered, 1974).

Serum T4 estimation appears to be an adequate discriminant of symptomatic hypothyroidism in the majority of patients. The low discriminant value of T3 estimation observed confirms earlier reports (Evered *et al*, 1973). Estimation of TSH remains the most sensitive index of primary hypothyroidism (Evered *et al*, 1973).

The diagnostic specificity of these techniques is satisfactory for routine purposes. The development of routine radioimmunoassay procedures for TSH estimation and the availability of synthetic TRH have made it possible to resolve diagnostic problems adequately.

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Schilddrüse 1975

Henning Symposium Publikation

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198 Abbildungen, 57 Tabellen

Georg Thieme Verlag Stuttgart 1977

Epidemiological Aspects of Thyroiditis and the Clinical Significance of Circulating Thyroid Antibodies

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Introduction

Circulating antibodies to various thyroid components may be found in patients with hypothyroidism, Hashimoto's disease, Graves' disease and also in a proportion of apparently normal people. The identification of these antibodies provides a marker of the presence of lymphocytic infiltration of the thyroid, and to a certain extent the titre of those antibodies reflects the degree of infiltration. It is clear that thyroid auto-antibodies may be associated with hypothyroidism and the work of many groups has indicated that autoimmune thyroiditis is the major cause of spontaneously occurring hypothyroidism in Caucasian races. Little, however, is known of the natural history of autoimmune thyroiditis or of the biological significance of this condition in its subclinical form (4). It has been suggested, from hospital-based studies, that the asymptomatic stages of this disease may be a significant risk factor for coronary artery disease.

A survey has been conducted in Whickham in the North East of England, to determine the prevalence of the different grades of hypothyroidism (4) and hyperthyroidism in the community, and to examine the possible relationship between subclinical hypothyroidism, hyperlipidaemia and ischaemic heart disease (1, 2, 6, 10).

Methods

Whickham is a mixed urban and rural area, whose adult population (20,000) is closely matched for age, sex and socio-economic group to the population of Great Britain as a whole. A sixth of the population was randomly selected from the electoral roll and 2,779 people (82.4 % available sample) were seen over a two year period. A questionnaire was completed by a member of the survey team and included a personal and family history of thyroid disease, cardiovascular disease, diabetes mellitus and pernicious anaemia. Each subject was examined for goitre and signs of thyroid disease. Blood pressure was recorded in the recumbent position using a random zero sphygmomanometer. A standard twelve lead electrocardiograph (ECG) was assessed using the Minnesota code (12). A fasting blood sample was taken for estimation of serum thyrotrophin (TSH) (9), thyroxine (T_4) (Thyopac-4® Radiochemical Centre, Amersham), triiodothyronine (T_3) (11), glucose, cholesterol (Technicon® method file N 24a), triglyceride (5) and lipoprotein electrophoresis. Thyroid cytoplasmic antibodies were

measured by fluorescence technique on serum diluted 1:10 (3) and thyroglobulin antibodies by a tanned red cell technique (7), a titre of 1:20 or more was considered to be significant.

Results

Hyperthyroidism was diagnosed at the survey in 7 people on the basis of clinical features, high T_4 and high T_3 levels. 37 people had been previously treated for hyperthyroidism and the diagnosis was confirmed in 25 from the original hospital records, the remaining 12 for whom no records were available being classified as "possible" hyperthyroidism. The overall prevalence of newly diagnosed and previously established hyperthyroidism was 1.2 % plus a further possible 0.45 %. Only three of these subjects were male giving a prevalence of 21 - 27/1,000 females and 1 - 2/1,000 males.

Overt Hypothyroidism was diagnosed at the survey in 5 people on the basis of clinical features, low T_4 and low T_3 levels; all had markedly raised TSH levels. 24 people had been previously diagnosed and treated for hypothyroidism and the diagnosis was confirmed in 17 from their original records, the remaining 7 for whom no records were available being classified as "possible" hypothyroidism. The overall prevalence of newly diagnosed and previously established overt hypothyroidism was 0.8 % plus a further 0.25 %. Only one subject was male, giving prevalence rates of 14 - 19/1,000 females and 1/1,000 males.

Thyroid cytoplasmic antibodies were present in 6.8 % population and thyroglobulin antibodies in 2 % population. Only 0.7 % population had thyroglobulin antibodies without cytoplasmic antibodies. Cytoplasmic antibodies are four times more common in women than in men and there is a marked increase in frequency from the time of the menopause. The frequency of visible goitre (6.9 % population; female : male 12 : 1), however, declines after the menopause and when the effect of age is eliminated there is no significant association between goitre and cytoplasmic antibodies.

Minor degrees of hypothyroidism. Serum TSH is not normally distributed, being markedly skewed to the right. 10 % of observations were below the limit of detection (< 0.5 mU/l), of the assay, the median value was 2.1 mU/l and 95 % observations were less than 6 mU/l which was defined as the upper limit of normal. Subjects with TSH > 6 mU/l, after exclusion of those with overt hypothyroidism, were shown to have significantly lower T_4 levels than subjects with normal TSH (by at least 1 µg/100 ml in each sex) but there was no significant difference in their respective T_3 levels. It was not possible to identify clinically these subjects who may be said to have a minor degree of hypothyroidism. The age and sex distribution of TSH in the total population and in the population excluding anyone with a history of thyroid disease, thyroid antibodies or goitre is shown in Figure 1.

TSH distribution in males does not vary significantly with age. The age-related increase in mean TSH in females is less evident when subjects with any marker of thyroid disease are excluded from the analysis. TSH was found to be significantly

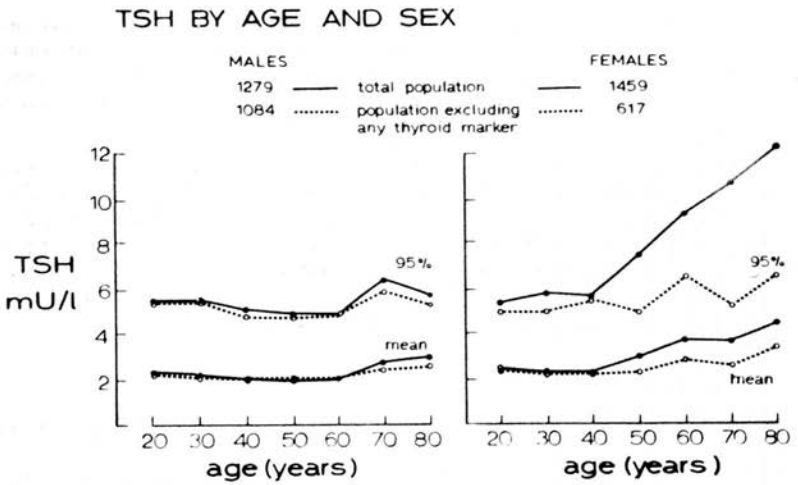

Figure 1: Age and sex distribution of serum TSH, mean and 95th percentile, for total population (1279 males, 1459 females) ————; population excluding any thyroid marker (1084 males, 617 females) -------

associated with antibodies but not with family history of thyroid disease or goitre. Two thirds of the population with high TSH have cytoplasmic antibodies, and the relative risk of having a high TSH in those with antibodies compared to those without antibodies is 20:1 in males and 13:1 in females.

Hypothyroidism, lipids and ischaemic heart disease. There were no significant differences in levels of cholesterol, triglyceride or lipoprotein patterns between those with or without thyroid antibodies, and those with or without a high TSH (excluding overt hypothyroidism) in either sex. There was no association between cytoplasmic antibodies or high TSH and a history or ECG evidence of ischaemic heart disease in males. There was a significant association between a raised serum TSH (but not thyroid antibodies) and a history of chest pain on effort and of minor, but not major, ECG changes, (Minnesota code 1.3, 4.2 or 4.3, 5.2 or 5.3, but not 1.1, 1.2, 4.1, 5.1, 6.1 or 7.1) in the females. The approximate mean relative risk of ECG changes and high TSH in women was shown to be 2, irrespective of age, cholesterol, triglyceride, systolic or diastolic blood pressure, ponderal index (weight/height^2) skinfold thickness or smoking habits.

Conclusions

Hyperthyroidism and overt hypothyroidism are each as common as diabetes mellitus which is found in approximately 1 % population. Thyroid cytoplasmic antibodies are found in 6.8 % population, and a TSH > 6 mU/l in 5 % population. 3 % population have both a high TSH and cytoplasmic antibodies. Thus nearly half of those with antibodies may be said to show a

minor degree of hypothyroidism, but the incidence of more severe hypothyroidism in such people remains to be determined. In a similar study in Finland overt hypothyroidism was much rarer, but an estimated 1.5 - 3 % population had both thyroglobulin antibodies and high TSH levels (8).

Cholesterol and triglyceride levels are shown to be unhelpful in the identification of minor degrees of hypothyroidism. There is an increased risk of minor ECG abnormalities in women with high TSH, but it cannot be stated with certainty that such ECG changes indicate ischaemic heart disease. This association is independent of other so-called risk factors for the development of coronary artery disease.

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Clinical Endocrinology (1977) 7, 481–493.

THE SPECTRUM OF THYROID DISEASE IN A COMMUNITY: THE WHICKHAM SURVEY

72

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(Received 20 January 1977; revised 29 June 1977; accepted 2 August 1977)

SUMMARY

A survey has been conducted in Whickham, County Durham, to determine the prevalence of thyroid disorders in the community. Two thousand seven hundred and seventy-nine people (82.4% of the available sample) were seen in the survey. The prevalence of overt hyperthyroidism was 19/1000 females rising to 27/1000 females when possible cases were included, compared with 1.6–2.3/1000 males. The prevalence of overt hyothyroidism was 14/1000 females rising to 19/1000 females when possible cases were included, compared with less than 1/1000 males. The prevalence of spontaneous overt hypothyroidism (excluding iatrogenic cases) was 10/1000 females or 15/1000 females including unconfirmed cases. Minor degrees of hypothyroidism were defined on the basis of elevated serum thyrotrophin (TSH) levels in the absence of obvious clinical features of hypothyroidism. TSH levels did not vary with age in males but increased markedly in females after the age of 45 years. The rise of TSH with age in females was virtually abolished when persons with thyroid antibodies were excluded from the sample. TSH levels above 6 mu/l were shown to reflect a significant lowering of circulating thyroxine levels and showed a strong association with thyroid antibodies in both sexes, independent of age. Elevated TSH levels (>6 mu/l) were recorded in 7.5% of females and 2.8% of males of all ages. Thyroglobulin antibodies were present in 2% of the sample. Thyroid cytoplasmic antibodies were present in 6.8% of the sample (females 10.3%, males 2.7%) and their frequency did not vary significantly with age in males but increased markedly in females over 45 years of age. 3% of the sample (females 5.1%, males 1.1%) had thyroid antibodies and elevated TSH levels and the relative risk of a high TSH level in subjects with antibodies was 20:1 for males and 13:1 for females, independent of age. Small goitres (palpable

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but not visible) were found in 8.6% of the sample and obvious goitres (palpable and visible) in 6.9%. Goitres were four times more common in females than in males and were most commonly found in younger rather than older females. TSH levels were slightly but not significantly lower in those with goitre than in those without goitre. There was a weak association between goitre and antibodies in females but not males.

There have been numerous studies of the prevalence of goitre but there is relatively little information on the prevalence of the whole range of autoimmune thyroid disorders in communities as opposed to selected hospital populations. The primary aim of the present study was to determine the prevalence of hyperthyroidism, the different grades of hypothyroidism, and autoimmune thyroiditis as well as goitre, using current diagnostic criteria, in a cross-section of a community.

METHODS

A survey was conducted in Whickham Urban District, a mixed urban and rural area in County Durham close to Newcastle-upon-Tyne. A systematic sample of the adult population was drawn from the electoral register published just prior to the survey which ran from July 1972 to June 1974. The first name was chosen at random and every sixth name was selected thereafter. Selected subjects were approached by their general practitioner, and if necessary a member of the survey team explained the purpose of the survey in detail. Substitutes were selected for those who had moved from the area. Participants were asked to attend the survey centre having fasted overnight.

The enquiry. Personal details were recorded and particular enquiry was made for: a personal or family history of goitre, hyperthyroidism and hypothyroidism with details of any investigations and treatment; possible symptoms and signs consistent with hypothyroidism or hyperthyroidism; diabetes mellitus and pernicious anaemia, current medication; smoking habits; pregnancy. Evidence of vascular disease was sought from a personal and family history of ischaemic heart disease, hypertension and cerebrovascular disease; a symptomatic enquiry for chest pain on effort, and possible myocardial infarction using a standard questionnaire (Rose & Blackburn, 1968), and intermittent claudication. Blood pressure was recorded using a random zero sphygmomanometer (Wright & Dore, 1970) with the patient in a recumbent position after completion of a standard twelve-lead electrocardiogram. A fasting blood sample was then taken for the following investigations: serum thyrotrophin (TSH) (Hall *et al.*, 1971), thyroxine (T4) (Thyopac-4, Amersham), triiodothyronine (T3) (Hesch & Evered, 1973), T3 Sephadex uptake (Thyopac-3, Amersham) used in conjunction with the T4 to derive a Free Thyroxine Index (Clark & Brown, 1970) (FTI), thyroglobulin antibodies (TGA) (Fulthorpe *et al.*, 1961), thyroid cytoplasmic (microsomal) antibodies by immunofluorescence (Doniach, 1967) and microsomal haemagglutination techniques (Bird & Stephenson, 1973); antibodies to gastric parietal cells, smooth muscle, mitochondrial and antinuclear factor were also measured by an immunofluorescence technique; cholesterol (Technicon method file N24a), triglyceride (Fletcher, 1968), and glucose (oxidase method, Autoanalyser); 24 h urinary iodine and serum inorganic iodine were estimated from single casual specimens of urine and serum (Vought *et al.*, 1963) in a sub-sample of participants. The effect of observer variation was analysed over the period of the survey but no important differences were found. Strict quality control was maintained on all laboratory investigations.

Data from the completed questionnaires were transferred to punch cards which were then processed using the Statistical Package for Social Sciences (Nie *et al.*, 1970).

RESULTS

The observed sample

A sample of 3538 people aged 18 years or over was drawn from the electoral register. A total of 168 people had died or left the area but their families were still in residence and no substitution was performed. The remaining available sample of 3370 included 284 people substituted from new households. 2284 people accepted the initial invitations from their general practitioners to participate in the survey. Members of the survey team personally contacted all but eighty-one of the remaining 1086 selected individuals of whom 495 were recruited into the survey. A total of 2779 people (82.4% of the available sample) took part in the survey. 89% of the participants were born in the counties of Durham and Northumberland, as were approximately 80% of their parents.

There were 1285 men (46%) and 1494 women (54%), mean (±SD) age 47.1±16.4 years, in the observed sample and their age distribution approximates to those of the populations of Whickham Urban District and Great Britain as a whole (O.P.C.S., 1971) but the sample has a smaller proportion of men and women under 25 years of age. The distribution of the sample by social class closely resembles that of Whickham but the latter has a slight preponderance of social classes I, II and III over classes IV and V compared with Great Britain as a whole.

There was a slightly higher proportion of women (56%) in the recruited sample, who were on average 2 years younger than the rest of the sample, but there was no significant difference in the frequencies of known thyroid or cardiovascular disease amongst the recruited and ready respondents. The sample was not further analysed by the degree of reluctance to participate and subsequent data are based on the total observed sample.

The prevalence of thyroid disorders

Hyperthyroidism. Seven women with clear clinical features of hyperthyroidism were identified in the survey; all had high T4 concentrations greater than 174 nmol/l* and correspondingly elevated free thyroxine indices.

Thirty-four women and three men had been previously diagnosed and treated for hyperthyroidism. The original records were traced for twenty-eight patients and the diagnosis was scored as: 'definite' in twenty-one women and one man whose records confirmed the appropriate clinical and laboratory features; 'probable' in two women and one man by criteria similar to those above except that the results of the laboratory tests which had been performed could not be traced; and 'possible' in eleven women and one man, in whom no records were available to confirm or refute the diagnosis although all gave a history compatible with hyperthyroidism prior to treatment. A further nine people who claimed to have had hyperthyroidism were excluded after examination of their original records.

The prevalence of established hyperthyroidism was, therefore 19/1000 females and 1.6/1000 males and if the possible cases were included was 27/1000 females and 2.3/1000 males. The prevalence of hyperthyroidism in the whole sample was 1.1% for established

*Reference range – mean ±SD 110.5±23.1 nmol/l).

cases rising to 1.6% when possible cases were included. The mean age at diagnosis was 48 years (range 25–70 years) and mean interval between diagnosis and participation in the survey was $7\frac{1}{2}$ years. Twenty-three women in whom the diagnosis was established had been treated during the previous 24 years, of whom eleven had been treated in the 6 years prior to the survey. (Diabetes mellitus, for comparison, had been previously diagnosed and treated in 0.8% of the sample and a further 1% had fasting glucose levels of >6 mmol/l.)

Overt hypothyroidism. Five women with clear clinical features of hypothyroidism were identified in the survey; all had low T4 concentrations, less than 46 nmol/l*, with corresponding low free-thyroxine indices and elevated TSH levels (16–77 mu/l).

Twenty-three women and one man had been previously diagnosed and treated for hypothyroidism. The original records were traced and the diagnosis scored as: 'definite' in fourteen women whose records confirmed appropriate clinical features, low thyroid hormone concentrations (PBI<236 nmol/l or T4<45 nmol/l) and a good response to thyroxine therapy; 'probable' in two women and one man on similar criteria except that the results of the diagnostic tests which had been performed could not be traced; 'possible' in seven women in whom a clinical diagnosis had been made but no thyroid function tests performed before treatment with thyroxine commenced. Four other people who claimed to have had hypothyroidism were not on treatment, had normal thyroid function tests and the diagnosis was excluded.

The prevalence of established overt hypothyroidism was 14/1000 females and less than 1/1000 males, and if the possible cases were included was 19/1000 females. Six women had been previously treated for hyperthyroidism and if these cases were excluded the prevalence of spontaneous overt hypothyroidism was 10/1000 females rising to 15/1000 females if possible cases were included. The prevalence of all established cases of overt hypothyroidism in the whole sample was 0.8% rising to 1.1% when possible cases were included. The mean age at diagnosis was 57 years (range 30–76 years) and the mean interval between diagnosis and participation in the survey was seven years.

Minor degrees of hypothyroidism were defined by a serum TSH>6 mu/l in the absence of obvious clinical features of hypothyroidism. The distribution of TSH was non-Gaussian, being markedly skewed to the right (Fig. 1). Ten per cent of observations were below the limits of detection of the assay (0.5 mu/l). TSH concentrations did not vary significantly with age in males (median 1.9 mu/l, mean 2.2 mu/l, 97.5 percentile 6.1 mu/l) and were similar in both sexes below 45 years of age but showed a marked rise in females above this age (median 2.2 mu/l, mean 2.9 mu/l, 97.5 percentile 10.5 mu/l).

All persons with a past or family history of thyroid disease, overt hypothyroidism, hyperthyroidism, goitre and thyroid antibodies were excluded from the sample and the remainder described as 'thyroid negative'. The distribution of TSH in the thyroid negative sample was not significantly different from that in the total sample for males and the marked rise in TSH in females over 45 years of age in the total sample was almost abolished in the thyroid negative sample (Fig. 2). The distribution of TSH concentrations in the thyroid negative sample was similar in males and females of all ages (median 2.0 mu/l, mean 2.2 mu/l, 97.5 percentile 6.0 mu/l) and the increment of mean TSH with age was less than 1 mu/l/100 years. Mean TSH concentrations were slightly but not significantly lower in persons with goitre than without goitre, and were not significantly different in those with

*Reference range – Mean ±SD 110.5±23.1 nmol/l.

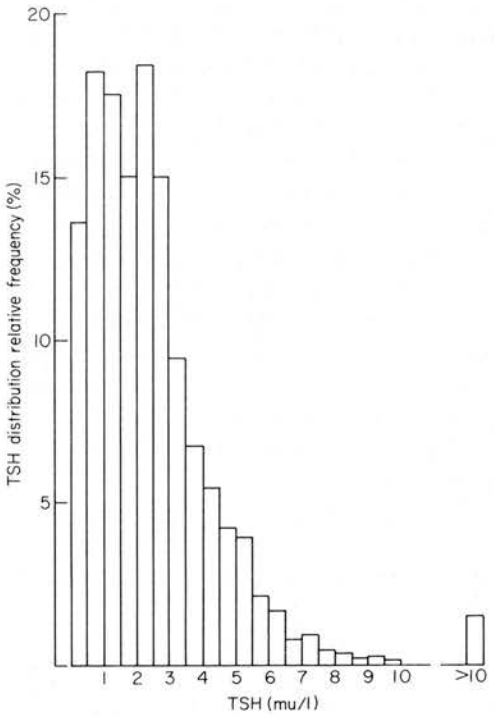

Fig. 1. TSH distribution: relative frequency in the total sample.

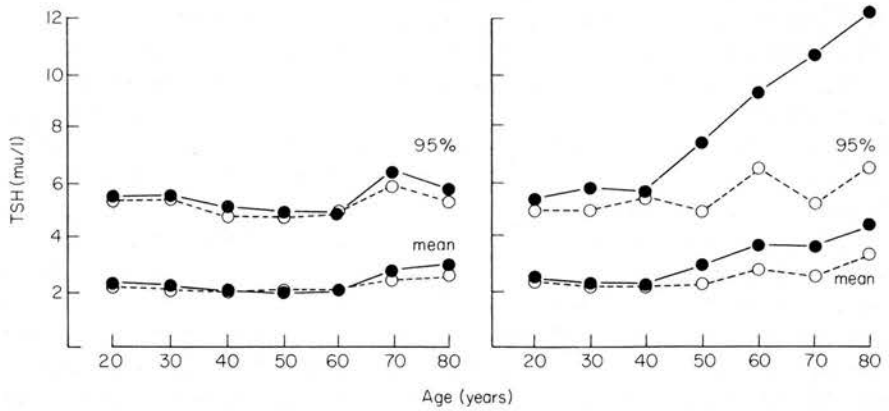

Fig. 2. TSH distribution by age and sex in the total sample (•) (1279 males and 1459 females) and in the sample excluding any other marker of thyroid disease (○) (1084 males and 617 females)

and without a family history of thyroid disease. Mean TSH concentrations were significantly higher in subjects with thyroid antibodies than in those without antibodies (TSH mean ±SE, males 5.7±0.9:2.1±0.1 mu/l, $P < 0.001$; females 7.3±0.9:2.4±0.1, $P < 0.001$). The rise in TSH in females was almost entirely due to the association between TSH and thyroid

antibodies. Mean T_4 concentrations (and FTI values) were significantly lower in subjects with a TSH>6 mu/l than in those with a TSH below this level (T4 mean±SE, males 89±3.9:102±1.3 nmol/l, $P<0.01$; females 95±2.6:111±1.3 nmol/l, $P<0.001$) but there was no significant difference in T_3 concentrations between the two groups in either sex.

TSH concentrations above 6 mu/l were nearly three times more common in females (7.5%) than in males (2.8%) and occurred most frequently in females over 45 years of age (Table 1).

Thyroid antibodies. Thyroglobulin antibodies (TGA) were present at a titre of 1:20 or more in 2% of the sample (females 3.0%, males 0.9%). Their frequency did not vary significantly with age in males but increased in females from less than 1% below the age of 45 years to 4.6% of the 45-54 year age group rising to 7.4% at age 75 and above (Table). Overall only 0.7% of the total sample had TGA without a positive immunofluorescence test for cytoplasmic antibodies.

Thyroid cytoplasmic antibodies (CYT) were scored as positive if present in serum diluted 1:10. Microsomal antibodies by micro-haemagglutination technique at a titre of 1:100 or more were present in 80% of sera giving positive results by immunofluorescence technique. CYT were present in 6.8% of the sample (females 10.3%, males 2.7%). The frequency of CYT did not vary significantly with age in males. The frequency of CYT in females ranged from 6.9–9.7% during the reproductive years, increasing to 13.7% at age 45–54 years and falling to 8.8% after 75 years of age (Table).

There was a strong association between CYT and gastric parietal cell antibodies (PCA) in both sexes. PCA were present in 20% of males with CYT compared to 4.3% without CYT ($P<0.001$) and in 21.4% of females with CYT compared to 6.9% without CYT ($P<0.001$). There was also an association between CYT and antinuclear factor (ANF) in females but not males: ANF was present in 13.4% of females with CYT compared to 3.6% of females without CYT ($P<0.001$) and in 2.6% of males with CYT compared with 2.0% without CYT (NS). There was no association in either sex between CYT and smooth muscle antibodies (males 1.6%, females 1.6%), antimitochondrial antibodies (males none; females 0.7%) or reticulin antibodies (males 2.2%, females 2.2%).

Thyroid cytoplasmic antibodies were present in 40% of males and 67% of females with a TSH>6 mu/l compared with 2.2% of males and 8% of females with normal TSH levels. Three per cent of the sample (females 5.1%, males 1.1%) had both thyroid antibodies and elevated TSH concentration. Overall 60% of subjects with TSH >6 mu/l and 80% of those with TSH >10 mu/l had thyroid antibodies. Conversely, nearly half the subjects with thyroid antibodies had a TSH >6 mu/l and the approximate mean relative risk of having a high TSH with respect to thyroid antibodies was 20:1 for males and 13:1 for females, independent of age.

The frequencies of thyroid antibodies and high TSH levels did not vary significantly with social class.

Goitre. Thyroid size was graded according to the palpability and visibility of the gland as follows: A, not visible and not palpable; B, not visible, palpable but of normal size and consistency; C, not visible, palpable and enlarged; D, visible with the neck extended and palpably enlarged (corresponding to WHO grade 1); E, visible on direct inspection with the neck in the normal position, palpation only needed to confirm that the swelling was due to the thyroid (corresponding to WHO grade 2 and above). The thyroid was not palpable in 61.4% of the sample (females 48.2%, males 76.6%), and palpable but normal (category B) in 23.1% of the sample F:M, 3:2). Small goitres (category C) were palpable in 8.6% of the

sample (F:M, 3:1) and obvious goitres (categories D + E) were observed in 6.9% of the sample (F:M, 13:1). Small and obvious goitres (C + D + E) were recorded in 15.5% of the sample (varying between 13.4% and 19.8% amongst regular observers).

The frequency of goitre in males (almost all category C) declined with age from 7.4% in those under 25 years of age to 3.8% in those aged 65–74 years and no goitres were detected in males over 75 years. Goitre occurred most commonly in women in the younger

Table 1. Age and sex distribution of selected thyroid abnormalities in the total sample (per cent of each age group). CYT, cytoplasmic immunofluorescence; MICRO, microsomal haemagglutination; TGA, thyroglobulin.

Age (years)	Number	TSH ⩾ 6 mu/l	Thyroid Antibodies			Goitre		Nodules
			CYT	Micro	TGA	Palpable not visible	Palpable + visible	
Males								
18-24	110	2.8	1.8	0.9	0	3.7	3.7	0
25-34	233	3.5	3.0	3.0	0.9	5.6	0.4	0
35-44	255	1.6	2.7	2.0	0.4	6.3	0.4	1.2
45-54	290	2.1	2.4	1.7	1.0	4.5	1.0	1.4
55-64	210	1.9	2.9	1.9	1.4	4.3	0	0.5
65-74	130	6.9	3.8	2.3	0.8	2.3	1.5	1.5
75+	57	3.5	1.8	1.8	1.8	0	0	0
All ages	1285	2.8	2.7	2.0	0.9	4.5	0.9	0.8
Females								
18-24	128	4.0	7.9	6.3	0	12.6	15.7	4.7
25-34	323	5.7	6.9	4.4	0.9	10.7	15.0	3.4
35-44	275	4.1	9.7	6.3	0.7	15.4	15.8	4.8
45-54	240	9.6	13.7	10.8	4.6	12.1	13.0	4.2
55-64	266	10.0	12.0	8.8	4.2	14.0	8.3	7.5
65-74	182	8.0	12.7	9.2	6.4	7.7	7.1	6.0
75+	80	17.4	8.8	8.8	7.4	9.1	3.9	9.1
All ages	1494	7.5	10.3	7.6	3.0	12.1	12.3	5.3
Total	2779	5.0	6.8	5.3	2.0	8.6	6.9	3.2

age groups varying between 25.7% and 31.2% in those under 45 years of age and declining to 12% of those over 75 years of age, mainly due to the reduced frequency of obvious (category D + E) rather than small (category C) goitres (Table 1).

Thyroid nodules were detected in only 0.8% of males but occurred in 5.3% of females and increased in frequency after the age of 45 years to 9.1% of women aged 75 years or more.

There was a lack of any significant association between goitre and thyroid antibodies in males. Goitre occurred in 36.5% of females with antibodies compared to 22.7% of those without antibodies and the approximate mean relative risk of having a goitre with respect to thyroid antibodies in females was 2.1 (95% confidence limits 1.4–2.9), independent of age. Estimations of the mean 24 h urinary iodine output and serum inorganic iodine based on

single casual specimens of urine and serum in a random subsample of 197 people showed no significant difference between those with and without goitre. Mean±SE urinary iodine output was 811±53 nmol/24 h, and mean±SE serum inorganic iodine was 21±1.2 nmol/l, and these results do not suggest iodine deficiency in the population.

DISCUSSION

The survey sample is shown to resemble closely the populations of Whickham and Great Britain as a whole in age, sex and social class distributions, although the sample slightly under-represents both sexes below the age of 25 years. The participants recruited into the survey were on average slightly younger than the other participants and could be said to represent the people missing from the survey but there is no way of representing those who were selected and died before they could be seen. There was no significant difference in the frequency of previously known thyroid disease between the recruited sample and the rest of the sample and thus analyses were based on the total sample. The average turnover of households during the survey (Gray *et al.*, 1950) and the fact that 89% of the participants and approximately 80% of their parents were born in Northumbria indicate that the community is fairly stable.

Cross sectional surveys allow the determination of prevalence data but give little indication of incidence or the natural history of disease states. Nevertheless, if the age of onset of an illness has been documented a rough estimate of its incidence can be derived if it is assumed that all the participants had been available over a given period. Such an assumption becomes less and less valid the longer the period under review owing to the mobility of the population and differential mortality associated with particular illnesses and is likely to lead to underestimation of the true incidence of any disorder.

The prevalence of hyperthyroidism in the present survey is at least tenfold higher than previous estimates based on hospital studies (Furszyfer *et al.*, 1970; Hoffenberg, 1974). It must, of course, be emphasized that a single prevalence rate merely provides a guide to the expected frequency. The range of prevalence rates from these data falls between 12 and 26 per 1000 for definite hyperthyroidism. Hospital records have limitations as an index of disease in a community as referral may be highly selective and incomplete, particularly if based on information from inpatients but not outpatients. Retrospective analyses of hospital records are also complicated by changes in diagnostic procedures which may have occurred during the period under review. The present prevalence data include patients who had been treated previously for hyperthyroidism although they were no longer hyperthyroid when seen at the survey. Too many assumptions concerning the availability of the same sample over a long period are required to justify a precise calculation of incidence but the number of cases diagnosed in the 6 years prior to the survey suggests an annual incidence of nearly two cases per thousand females. The true annual incidence is likely to lie between 2 and 3 cases per 1000 females, allowing for the mobility of the population and for the possible cases whose diagnosis could not be confirmed because of the lack of their original records. The number of new cases detected during the survey (4.5/1000 females), whilst not representing annual incidence as the survey took 2 years to complete and the sample was not examined at the beginning and end of this period, is not inconsistent with the above estimate of annual incidence, allowing also that some of the cases were detected at an early stage.

The prevalence of overt hypothyroidism is less than that of hyperthyroidism, particu-

larly if the iatrogenic cases resulting from treatment of the latter condition are excluded. Overt hypothyroidism was diagnosed at the survey in five females (3.3/1000 females) and this compares with 2/1000 of the sample (males and females) tested in a community study in Finland (Gordin *et al.*, 1972). The number of cases diagnosed in the 6 years prior to the survey, with the same reservations as were applied to the data on hyperthyroidism, suggest an annual incidence of between 1 and 2 cases per 1000 females.

The seven subjects with untreated hyperthyroidism and the five with untreated hypothyroidism at the survey were identified by the presence of clear clinical features of these conditions, and the laboratory investigations, which fell well outside the reference ranges in each case, were merely used to confirm the diagnosis. The diagnosis of untreated thyroid disease was only accepted in those patients whose records contained an unequivocal description of the appropriate clinical features supported by clearly abnormal thyroid function tests.

Lesser degrees of hypothyroidism were identified using the sensitive marker provided by TSH radioimmunoassay. TSH concentrations cannot be expressed in logarithmic terms as 10% of observations were below the limit of detection of the assay, nor does its skewed distribution strictly permit description in terms of a mean and standard deviation although this was used for comparison of TSH levels between sub-groups. 95% of TSH estimations were below 6 mu/l and a TSH >6 mu/l was shown to reflect a significant reduction in T_4 but not T_3 levels, and to have a strong association with thyroid antibodies independent of age. People with a TSH level >6 mu/l, excluding overtly hypothyroid subjects, were therefore defined as having a minor degree of hypothyroidism. All the overtly hypothyroid subjects had several symptoms and signs but in the rest of the sample no recorded clinical feature, singly or in combination, could be consistently identified with any one thyroid function test including TSH. The term minor degrees of hypothyroidism has therefore been used to embrace the sub-groups of mild and subclinical hypothyroidism proposed by Evered & Hall (1972).

Asymptomatic patients with thyroid antibodies have previously been shown to have reduced exchangeable iodine pools and abnormal responses to exogenous TSH stimulation (Buchanan *et al.*, 1965) and serum TSH concentrations estimated by bioassay have also been shown to be higher in patients with thyroid antibodies than in those without antibodies (Bonnyns & Bastenie, 1966). In the present study mean TSH concentrations were markedly elevated in subjects with thyroid antibodies and 3% of the sample had thyroid antibodies and TSH levels $\geqslant$6 mu/l. Gordin *et al.* (1972) found that between 1.5% and 3% of their samples of approximately 3000 people from two populations in Finland had high thyroglobulin antibody titres and raised TSH concentrations. A study of approximately 5000 people including factory workers, physicians and blood donors in the USA (Lowrey & Starr, 1959) using PBI as a screening test, suggested a higher prevalence of all grades of hypothyroidism but the PBI is a relatively insensitive test of thyroid function. It is impossible to determine the possible bias of the selection procedure, and only a small selected subsample were tested for other evidence of thyroid disease.

Changes in techniques used to measure thyroid antibodies limit comparisons between different studies. Thyroglobulin antibodies were found less frequently and in lower titres than in many previous community studies (Gordin *et al.*, 1972; Dingle *et al.*, 1966) and this may be largely attributable to refinements in the microapparatus and reagents used (Bird & Stephenson, 1973). The prevalence of thyroid cytoplasmic (microsomal) antibodies measured by immunofluorescence technique in Whickham is similar to that found

in Carterton, New Zealand (Couchman *et al.*, 1970). Microsomal antibodies detected by the micro-haemagglutination technique were present in 80% of sera positive by immunofluorescence technique and the former test may supersede the complement fixation test because of its greater precision and reproducibility (Bird & Stephenson, 1973). The increased frequency of thyroid antibodies in post-menopausal women is consistent with previous observations irrespective of the techniques used to measure antibodies but no new evidence is available to explain this phenomenon. Gastric parietal cell antibodies show a similar age and sex distribution to that of thyroid cytoplasmic antibodies and the previously demonstrated association of these antibodies (Irvine, 1965, 1974) is confirmed by the present data.

Thyroid cytoplasmic antibodies and elevated TSH levels were twice as common in women over the age of 45 years as in those under this age, thus over 10% of post-menopausal women had evidence of autoimmune thyroiditis and a minor degree of hypothyroidism, although only 0.5% had overt hypothyroidism diagnosed at the survey. Similar figures pertained to women over 65 years of age. Geriatric inpatient enquiries might be expected to reveal a higher proportion of patients with varying degrees of hypothyroidism but hospitalized geriatric patients are a highly selected group whose thyroid function tests may be disturbed by intercurrent non-thyroid illness, with or without changes in thyroid hormone binding proteins (Oppenheimer, 1968; Jeffreys *et al.*, 1972; Burger *et al.*, 1976).

Thyroid size was graded by a modification of the criteria of Perez *et al.* (1960) which allowed the recognition of a thyroid that was palpably enlarged but not visible as a small goitre. Grading of thyroid size is inherently subjective and it is necessary to decide between goitre and non-goitre (MacLennan *et al.*, 1969). The distinction of a palpable but invisible goitre from a palpable normal thyroid is not easy and accounts for some of the observer variation. Although slightly different criteria for goitre have been used by other authors (Kilpatrick *et al.*, 1963; Dingle *et al.*, 1966), all define a thyroid that is visible as well as palpable as a goitre. The frequency of obvious goitre in Whickham is similar to that found in New Herrington, County Durham (Dingle *et al.*, 1966), slightly greater than in Ormiston, East Lothian and much less than in Sheffield, Yorkshire (Kilpatrick *et al.*, 1963). The method of estimation of daily urinary iodine output and serum inorganic iodine (SII) from single casual specimens of urine and serum (Vought *et al.*, 1963) is relatively crude but has been shown to give similar results to those obtained by more sophisticated isotope dilution techniques (Boyle *et al.*, 1965). The mean 24 h urinary iodine and SII levels in the subsample tested do not indicate that Whickham is an iodine deficient area. The age distribution of goitre in women is in marked contrast to the distribution of thyroid antibodies although the age distribution of thyroid nodules more closely resembles that of thyroid antibodies. The association between goitre and thyroid antibodies in women was independent of age but was of a relatively low magnitude (mean relative risk 2) compared with the association of antibodies and high TSH levels (mean relative risk 13) and there was a lack of association between goitre and TSH. Thyroid antibodies are found in most cases of Hashimoto's goitre which are relatively uncommon but the majority of so-called 'simple goitres' may not have any association with autoimmune thyroiditis and their pathogenesis is not established.

There is clearly a large reservoir of autoimmune thyroid disease in the community and clinically obvious hyperthyroidism and hypothyroidism are only the extremes of the spectrum. The proportion of the sample with sub-clinical hyperthyroidism (Hall *et al.*, 1973) is unknown as the TRH test (Ormston *et al.*, 1971) which can be used to detect thyroid autonomy was not part of the screening procedures adopted for the survey. Nearly

half the sample with thyroid antibodies had high basal TSH levels possibly indicating a minor degree of hypothyroidism. It is postulated that more severe degrees of hypothyroidism will eventually ensue in people with autoimmune thyroiditis but the rate of this development remains to be established and is the subject of further longitudinal studies. Evidence from patients previously treated for hyperthyroidism by radioiodine (Tunbridge *et al.*, 1974; Toft *et al.*, 1975) or partial thyroidectomy (Evered *et al.*, 1975) indicates that the process of decompensation from minor to overt hypothyroidism may take many years. Gordin & Lamberg (1975) have recently reported that five out of eighteen subjects with symptomless autoimmune thyroiditis developed overt hypothyroidism during a mean follow up of 3 years. Four of their five subjects who became overtly hypothyroid had markedly elevated TSH levels (>19.5 mu/l) when initially assessed. Such rapid deterioration of thyroid function is unlikely to occur in the majority of subjects with autoimmune thyroiditis.

Lymphocytic infiltration of the thyroid, which has been shown to be associated with the presence of circulating thyroid antibodies during life, has been found at post mortem in a high proportion of patients dying from non-thyroid disease (Goudie *et al.*, 1959; Bastenie *et al.*, 1967; Williams & Doniach, 1962). The possible association between minor degrees of hypothyroidism, autoimmune thyroiditis, lipid disorders and ischaemic heart disease will be examined in subsequent communications. Whilst some subjects with minor degrees of hypothyroidism may develop overt hypothyroidism in due course (which would then require treatment) prophylactic treatment with thyroxine may not be without hazard and would present formidable logistic problems to patients and medical staff because of the careful supervision that would be necessary. The authors feel that until further evidence of the incidence of overt hypothyroidism becomes available the indiscriminate introduction of thyroxine therapy in asymptomatic patients with slightly elevated TSH levels is not justified.

ACKNOWLEDGMENTS

The authors thank the people of Whickham for participating in the survey; the General Practitioners of Whickham and Dunston and their receptionists for their cooperation; the staff of Whickham Cottage Hospital and Dunston Health Centre for their hospitality; the laboratory staff of the Departments of Medicine and Clinical Biochemistry, Royal Victoria Infirmary, and the Department of Haematology, Newcastle General Hospital for their collaboration; and the successive secretaries to the survey – Kay Bruder, Anna Micklethwaite, Valerie Parrish and Elke Clark – for their invaluable help. Financial support was gratefully received from the Department of Health and Social Services, the League of Friends of the Royal Victoria Infirmary, Newcastle-upon-Tyne, the Radiochemical Centre, Amersham, Mallinkrodt Ltd, Glaxo Ltd, and Roche Products Limited.

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Clinical Endocrinology (1977) 7, 495–508.

LIPID PROFILES AND CARDIOVASCULAR DISEASE IN THE WHICKHAM AREA WITH PARTICULAR REFERENCE TO THYROID FAILURE

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(Received 20 January 1977; revised 29 June 1977; accepted 2 August 1977)

SUMMARY

The age and sex distribution of ischaemic heart disease (IHD), other vascular disorders and serum lipid concentrations and the possible association between these factors and thyroid failure have been examined in a community survey. A past history of IHD occurred in 7.5% of males and 4.8% of females. 'Chest pain on effort' was found in 7.4% of males and 7.8% of females and 'possible infarction' in 5.4% of males and 3.8% of females. Major ECG changes were found in 4.7% of both sexes and minor changes in 6.5% of males and 11% of females. Intermittent claudication was present in 4.8% and cerebrovascular accidents had occurred in 2% of the sample. Diastolic blood pressures greater than 100 mmHg were found in 13% of all males and 11% of all females. Cholesterol concentrations were normally distributed. Mean cholesterol rose by approximately 0.25 mmol/l/decade in both sexes to reach a maximum in the 55–64 years age group and declined slightly thereafter. Triglyceride values were skewed to the right and increased by 0.2 mmol/l/decade in males up to the 45–54 years age group and by 0.1 mmol/l/decade in females to a peak in the 65–74 years age group – and declined after these ages. Electrophoresis revealed Type IIa patterns in 3% of males and 9% of females, IIb in 1% of both sexes and Type IV in 13% of males and 3% of females. There was no association in males between IHD and thyroid antibodies or minor degrees of thyroid failure. There was a weak association between minor ECG changes and minor degrees of hypothyroidism (but not thyroid antibodies) in females which was independent of other variables. The significance of this observation depends upon the interpretation of the ECG abnormalities and will only be established by longitudinal studies. No association was noted between lipid concentrations and thyroid antibodies or minor degrees of hypothyroidism.

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Ischaemic heart disease (IHD) and hyperlipidaemia are common in Western society and it has been shown that increasing cholesterol and triglyceride concentrations are important risk factors for IHD (Keyes *et al.*, 1963; Carlson & Bottiger, 1972; Rosenman *et al.*, 1970). Other factors known to be associated with IHD include blood pressure, obesity, smoking habits and lack of exercise. It has been suggested that asymptomatic auto-immune thyroiditis may be an additional risk factor for the development of IHD (Bastenie *et al.*, 1967, 1971). Overt hypothyroidism may be associated with hypercholesterolaemia (Miettinen, 1968) and hypertriglyceridaemia (Tulloch *et al.*, 1973) and some authors have suggested that hypercholesterolaemia may precede other evidence of incipient hypothyroidism (Fowler & Swale, 1967; Fowler *et al.*, 1970). The aims of this study are to describe the prevalence of IHD, other vascular disorders and hyperlipidaemia in the Whickham population and to examine the possible relationship between these phenomena and auto-immune thyroiditis and minor degrees of hypothyroidism.

METHODS

The survey methods have been described previously (Tunbridge *et al.*, 1977). 2779 subjects were seen in this study (82.4% of the available sample) and the age, sex and social class structure of the observed sample closely matched that of Great Britain as a whole. A personal and family history of thyroid disease, cardiovascular disease, diabetes mellitus, pernicious anaemia, smoking habits and medication was obtained from each subject. Blood pressure was recorded with the patient recumbent using a random zero sphygmomanometer (Wright & Dore, 1970). Height, weight, pulse rate and the presence of arcus (partial, involving upper and lower limbus or circumferential) or xanthelasma were recorded at physical examination. Subscapular and mid-triceps skinfold thickness were measured using Harpenden calipers.

Ischaemic heart disease (IHD). Evidence of IHD was sought in three ways: enquiry for a past history of angina or myocardial infarction, a standard questionnaire relating to chest pain and examination of the electrocardiogram (ECG). (a) Past history. Patients were asked directly if they had ever had angina or a myocardial infarction. The answer was only scored as positive if the diagnosis had been made by the general practitioner or after hospital investigation. (b) Standard questionnaire (Rose & Blackburn, 1968). 'Chest pain on effort' was scored as positive when the patient admitted to central or left chest pain which was relieved by rest or nitroglycerine. 'Possible infarction' was scored as positive if the subject gave a history of severe pain across the front of the chest lasting at least half an hour on one or more occasions. (c) Electrocardiogram. A standard 12 lead ECG was recorded on each subject and classified according to the Minnesota code (Rose & Blackburn, 1968) by one observer (JGE) without reference to the above criteria. 'Major items' included pronounced Q waves, major S-T segment depression (>1.0 mm) and or major T wave inversion (>5.0 mm) complete heart block or complete left bundle branch block (Minnesota codes 1.1 or 1.2, 4.1, 5.1, 6.1 or 7.1). 'Minor items' after exclusion of major items, included borderline Q waves, minor S-T depression (0.5–1.0 mm) and minor T wave depression (0–5 mm) (Minnesota codes 1.3, 4.2 or 4.3, 5.2 or 5.3).

Combination of criteria. Patients with 'chest pain on effort' and/or 'major ECG items' were classified as having 'probable heart disease'. Subjects with 'possible infarction' and/or 'minor ECG items', after exclusion of those with probable heart disease were grouped as 'possible heart disease'.

Hyperlipidaemia. Participants had fasted for 10–12 h before attending the survey. A

venous sample was obtained from each subject and cholesterol (Technicon® method file N24a) and triglyceride (Fletcher, 1968) estimated. Lipoprotein electrophoresis on agarose was carried out on each subject in whom the serum cholesterol was greater than 6.7 mmol/l or the triglyceride was greater than 1.8 mmol/l. Lipoprotein types were classified according to the modified Fredrickson classification (Fredrickson *et al.*, 1967; Beaumont *et al.*, 1970).

Other investigations. Other investigations on the fasting serum included glucose, thyrotrophin (TSH), thyroxine, triiodothyronine, T3 sephadex uptake, thyroglobulin and thyroid microsomal antibodies. The methods have been described in an earlier paper (Tunbridge *et al.*, 1977).

Statistical analysis. Data were analysed using the Statistical Package for the Social Sciences (Nie *et al.*, 1970). Association between variables was tested using the method of approximate mean relative risk (MRR) (Armitage, 1971).

RESULTS

Ischaemic heart disease

The age and sex distribution of IHD using the above criteria are shown in Table 1. ECG items (major and minor) showed a stronger association with a past history of IHD (MRR 5.9) than with a positive response to the standard questionnaire on 'Chest pain on effort' (MRR 2.6) or 'possible infarction' (MRR 3.9). Considerable variations in sensitivity (44–100%) and specificity (64–100%) was noted between observers when tested against a set of standard histories (Rose & Colley, 1969). The various criteria of IHD have, therefore, been considered separately in subsequent analyses.

Other evidence of vascular disease

Intermittent claudication was recorded in 4.8% of the sample and was as common in males as females at all ages. It occurred most frequently in males aged 65–74 years (17%) and in females over this age (15%). Cerebrovascular accidents had occurred in 2% of the sample and were more common in males over 65 years (10-12%) than in females of comparable age (4-8%).

Blood pressure (BP)

The distribution and levels of systolic and diastolic blood pressure are shown in Table 2. The distribution of systolic BP is skewed to the right while the distribution of diastolic BP was normal. It was noted that mean systolic pressure rose approximately 8 mmHg/decade in males from the age of 45 years and 11 mmHg/decade in females from the age of 35 years. Mean diastolic pressures rose in males to reach a plateau after 45 years while there was a progressive rise in females to the age of 65 years.

Cholesterol

The age and sex distribution of cholesterol is shown in Table 3. The values were normally distributed and were significantly lower in males than females ($p < 0.001$). The disproportionate frequency of high cholesterol values in women under the age of 35 is in part due to the influence of pregnancy and oral contraceptives. Thus in the 18–24 age group the mean (± SEM) cholesterol of 7 pregnant women was 7.8 ± 1.06 and 5.6 ± 0.18 mmol/l in 45 women on an oral contraceptive. Comparable figures for the 25–34 age group were 6.2 ± 0.34 and 5.8 ± 0.10 mmol/l for 18 pregnant women and 86 taking an oral contraceptive

Table 1. Ischaemic heart disease by varying criteria: age and sex distribution (per cent of age group)

Age	Number	Past history A	Chest pain on effort B	Possible infarction C	Major ECG changes D	Minor ECG changes E	Probable IHD (B ± D)	Possible excluding probable IHD (C ± E) — (B ± D)
Males								
18–24	110	0	1.8	0.9	0.9	0	2.7	0
25–34	233	0.4	1.3	1.7	0.4	1.7	1.7	3.4
35–44	255	1.6	2.0	3.5	0.4	1.6	2.4	3.5
45–54	290	4.5	6.6	3.4	3.1	3.8	8.3	4.5
55–64	210	16.2	13.3	11.4	7.6	14.3	19.5	13.3
65–74	130	28.5	24.6	14.6	14.6	17.7	35.4	12.3
75+	57	14.0	10.5	5.3	24.6	19.3	33.3	15.8
All male	1285	7.5	7.4	5.4	4.7	6.5	11.1	6.5
Females								
18–24	128	0	0.8	0.8	0	3.9	0.8	4.7
25–34	323	0	0.9	1.9	0.9	4.0	1.9	5.3
35–44	275	1.5	2.5	2.5	1.5	7.3	3.6	8.4
45–54	240	2.9	8.4	2.9	3.7	10.8	11.7	10.4
55–64	266	6.8	13.2	4.9	6.4	15.8	16.9	12.8
65–74	182	13.7	20.9	9.3	11.5	23.1	26.9	19.2
75+	80	22.5	16.2	7.5	20.0	20.0	28.8	18.8
All female	1494	4.8	7.8	3.8	4.7	11.0	10.8	10.4

Table 2. Blood pressure: age and sex distribution of systolic and diastolic BP, mean ± SD and 95th percentile (mmHg)

Age	Number	Systolic BP (mmHg)			Diastolic BP (mmHg)		
		Mean	SD	95th percentile	Mean	SD	95th percentile
Males							
18–24	109	134	14	154	79	11	93
25–34	233	135	14	156	84	12	100
35–44	255	136	14	160	89	10	104
45–54	288	144	12	180	92	12	111
55–64	209	154	23	194	94	13	113
65–74	130	164	23	204	94	13	115
75+	57	169	24	200	93	16	125
All ages	1281	144	22	185	89	13	109
Females							
18–24	126	121	11	139	74	11	89
25–34	320	121	14	142	76	10	91
35–44	272	129	17	159	82	11	100
45–54	240	140	21	176	87	13	107
55–64	264	158	25	196	93	13	115
65–74	178	170	27	218	95	14	116
75+	76	175	26	230	95	16	123
All ages	1476	141	28	192	85	14	108

respectively. These values compare with means of 5.4 ± 0.13 and 5.4 ± 0.05 mmol/l respectively for the total in each age group. There was a progressive rise in mean serum cholesterol concentration in both sexes to the age of 65 but a small decline thereafter.

Triglycerides

The age and sex distribution of triglycerides is shown in Table 4. It was noted that triglyceride values were positively skewed in the sample studied. The mean values were higher in males than females ($P < 0.001$). Mean levels increased with age in males up to the 45–54 years age group and declined thereafter (although a further small rise was noted over the age of 75 years). Mean levels in females increased at a slower rate than males to a peak in the 65–74 years age group. Pregnancy and oral contraceptive medication also had a major effect on triglyceride concentration in the younger women. The mean triglyceride concentration was 1.8 ± 0.26 in seven pregnant women and 1.1 ± 0.06 mmol/l in forty-five on an oral contraceptive in the 18–24 years age group, and 1.5 ± 0.19 in eighteen pregnant women and 1.2 ± 0.04 mmol/l in eighty-six women on an oral contraceptive in the 25–34 years age group. These values compare with 0.9 ± 0.04 and 0.9 ± 0.03 mmol/l, respectively, for the total in each age group.

Lipoproteins

Lipoprotein electrophoresis was performed on one-third of the total sample. The frequency of abnormal lipoprotein patterns increased with increasing levels of cholesterol and triglyceride. A Type IIa pattern was found in only 0.9% of subjects with a cholesterol less than 6.7 mmol/l, but in 13% of those with values between 6.7 and 7.8 mmol/l and in 48% of

Table 3. Cholesterol (mmol/l): age and sex percentiles

Age	Number	Mean	SD	Percentiles						
				2.5	5	10	50	90	95	97.5
Males										
18–24	109	4.8	0.9	3.3	3.4	3.8	4.7	5.8	6.6	7.1
25–34	231	5.4	0.9	3.7	4.1	4.2	5.3	6.6	7.0	7.6
35–44	255	5.8	1.0	4.0	4.2	4.6	5.7	7.0	7.4	7.9
45–54	288	6.0	1.2	3.9	4.0	4.5	5.9	7.3	7.7	8.2
55–64	208	6.0	1.0	4.2	4.5	4.7	5.9	7.4	7.8	8.1
65–74	130	5.7	1.0	4.0	4.2	4.5	5.6	7.0	7.6	7.8
75+	57	5.6	1.1	3.7	3.9	4.2	5.6	6.9	7.5	7.8
All male	1278	5.7	1.1	3.8	4.0	4.4	5.6	7.1	7.5	7.9
Females										
18–24	127	5.4	1.5	3.4	3.6	4.1	5.2	6.7	7.6	9.1
25–34	319	5.4	1.0	3.9	4.1	4.4	5.3	6.7	7.4	8.0
35–44	270	5.8	1.0	4.1	4.4	4.6	5.7	6.9	7.5	7.7
45–54	240	6.2	1.0	4.5	4.7	5.0	6.1	7.5	8.2	8.5
55–64	261	6.7	1.3	4.6	4.9	5.2	6.6	8.2	8.9	9.4
65–74	173	6.7	1.3	4.4	4.6	4.9	6.7	8.2	9.1	9.7
75+	69	6.3	1.3	3.8	4.4	4.7	6.0	8.2	8.6	8.8
All female	1459	6.0	1.3	4.1	4.4	4.6	5.9	7.6	8.2	8.9
Total	2737	5.8	1.2	3.9	4.2	4.5	5.8	7.4	7.9	8.5

Table 4. Triglyceride (mmol/l): age and sex percentile

Age	Number	Mean	SD	Percentiles						
				2.5	5	10	50	90	95	97.5
Males										
18–24	109	0.91	0.63	0.34	0.37	0.44	0.75	1.42	1.75	2.75
25–34	231	1.36	0.87	0.41	0.52	0.63	1.14	2.49	3.20	3.94
35–44	255	1.51	0.95	0.30	0.55	0.68	1.33	2.63	3.27	3.76
45–54	288	1.58	1.13	0.49	0.56	0.65	1.27	2.81	3.45	5.16
55–64	208	1.44	0.84	0.45	0.53	0.65	1.22	2.47	3.20	3.54
65–74	130	1.27	0.63	0.50	0.56	0.63	1.09	2.12	2.52	2.77
75+	57	1.36	0.90	0.45	0.53	0.59	1.19	2.30	3.37	3.87
All male	1278	1.41	0.92	0.43	0.50	0.61	1.16	2.50	3.15	3.89
Females										
18–24	127	0.87	0.44	0.35	0.40	0.47	0.75	1.49	1.77	2.21
25–34	319	0.88	0.47	0.29	0.35	0.42	0.76	1.45	1.80	2.18
35–44	270	0.97	0.65	0.31	0.38	0.46	0.83	1.52	2.06	2.48
45–54	240	1.09	0.58	0.45	0.48	0.55	0.98	1.79	2.09	2.57
55–64	261	1.26	0.77	0.48	0.55	0.63	1.12	2.02	2.40	2.85
65–74	173	1.32	0.63	0.52	0.61	0.70	1.17	2.11	2.61	3.06
75+	69	1.28	0.58	0.51	0.59	0.67	1.16	2.12	2.31	2.48
All female	1459	1.07	0.63	0.36	0.42	0.49	0.82	1.78	2.18	2.55
Total	2737	1.23	0.80	0.38	0.45	0.54	1.02	2.11	2.70	3.32

subjects with values greater than 7.8 mmol/l. Similarly, a Type IV pattern was only found in 0.4% of subjects with triglyceride values less than 1.8 mmol/l, but in 30% of subjects with values between 1.8 and 2.26 mmol/l and in 66% with values above this level. It is estimated that excluding subjects with cholesterol levels below 6.7 mmol/l and triglyceride levels below 1.8 mmol/l from routine electrophoresis, that approximately twenty-eight subjects (~ 1% of the sample) with abnormal lipoprotein patterns were not studied. Normal lipoprotein patterns were estimated to occur in 80% of males and 85% of females. The age and sex distribution of the various lipoprotein types is described in Table 5.

Table 5. Hyperlipidaemia: frequency (as a percentage of each group) of major abnormal lipoprotein types

		Lipoprotein types			
Age	Number	IIa	IIb	IV	V
Males					
18–24	110	2	0	3	2
25–34	233	1	1	12	2
35–44	255	3	1	15	1
45–54	290	3	1	17	1
55–64	210	5	1	12	1.5
65–74	130	1	1	9	0
75+	57	2	0	6	3.5
All ages	1285	3	1	13	1.5
Females					
18–24	128	4	1	0	0
25–34	323	3	1	3	0
35–44	275	3	1	3	0.5
45–54	240	10	0	3	0.5
55–64	266	15	1	5	1
65–74	182	15	1	7	0.5
75+	80	7	4	4	2.5
All ages	1494	9	1	3	0.5

Xanthelasma and arcus

Xanthelasma were found in 1.1% of all males (none under 45 years, 2.0% over 45 years), and 1.3% of all females (0.1% under 45 and 2.3% over 45 years). There were no significant differences in mean cholesterol or triglyceride levels of those with xanthelasma compared to age and sex matched controls.

Partial arcus was observed in 14% of all males (22% over 45 years) and 10% of females (18% over 45 years). Complete arcus was seen in 18% of males and 10% of females. There was a highly significant increase in cholesterol in those with partial or complete arcus. There was no correlation with serum triglyceride levels.

Cardiovascular disease and autoimmune thyroiditis

Males with antibodies were compared to those without antibodies and there was no significant difference in age, or systolic BP but their diastolic pressure was slightly raised. There was no difference in the frequency of IHD by any criteria, intermittent claudication or

cerebrovascular disease (Table 6). There was a positive association between thyroid antibodies and age, systolic and diastolic BP, and 'chest pain on effort' in the females. There was, however, no association between thyroid antibodies and the more objective criteria of IHD (Table 6).

Table 6. Thyroid antibodies and vascular disease

Variable	Males				Females			
	Thyroid Antibodies		t	P	Thyroid Antibodies		t	P
	Present	Absent			Present	Absent		
Number	40	1200			149	1200		
Age (years) mean ± SE	49.1 ± 2.6	46.5 ± 0.5	0.97	NS	49.7 ± 1.3	46.2 ± 0.5	2.55	< 0.05
Systolic BP mean ± SE (mmHg)	150 ± 3.6	143 ± 0.6	1.90	NS	143 ± 2.2	138 ± 0.8	2.25	< 0.05
Diastolic BP mean ± SE (mmHg)	94 ± 2.2	88 ± 0.4	2.31	< 0.05	86 ± 1.2	83 ± 0.4	2.11	< 0.05
History I.H.D. (%)	7.5	7.7	*	NS	5.4	4.5	*	NS
Chest pain on effort (%)	7.5	7.1	*	NS	12.2	6.6	*	< 0.05
Possible infarction (%)	5.0	5.5	*	NS	5.4	3.7	*	NS
ECG minor (%)	7.5	6.5	*	NS	11.4	10.6	*	NS
ECG major (%)	5.0	4.6	*	NS	7.4	4.2	*	NS
Int. claudication (%)	2.5	4.5	*	NS	9.5	3.5	*	< 0.01
C.V.A. (%)	0	2.2	*	NS	0.7	1.7	*	NS

* Binomial test.

Cardiovascular disease and minor degrees of hypothyroidism

No analysis of the data with respect to overt hypothyroidism was possible at the time of survey, since the majority of patients were on replacement therapy. Those patients (five) who were identified as having overt hypothyroidism at the survey were excluded from this analysis.

There was no association in males between a minor degree of hypothyroidism, (TSH > 6 mu/l; Tunbridge *et al.*, 1977) and BP, IHD or other vascular disease (Table 7). Females showed a positive association between a raised TSH and age, systolic and diastolic BP, 'chest pain on effort', 'possible infarction', minor (but not major) ECG items and intermittent claudication.

These data were further analysed in the females, but only the more objective criteria of IHD (i.e. ECG items) were used because of observer variation in the use of the standard questionnaire. The relatively small number of subjects with high TSH and ECG items did not permit separate statistical analysis for each group and thus subjects were grouped into three age groups (18–44, 45–64, 65 years and over). Lack of heterogeneity between the relative risks in each age division (Woolf's test) allowed the expression of MRR for the group as a whole. The association of ECG abnormalities and high TSH was examined in relation to cholesterol, triglyceride, systolic and diastolic BP, Quetelet's index (weight/height^2), skinfold thickness and smoking habits.

ECG changes and high TSH were shown to have an approximate MRR of 2.2 (95% confidence limits 1.4–3.9) irrespective of age and independently of any of the above variables.

Table 7. Serum thyrotrophin (TSH) levels and vascular disease

Variable	Males				Females			
	TSH (mu/l)		*t*	*P*	TSH (mu/l)		*t*	*P*
	⩾ 6	< 6			⩾ 6	< 6		
Number	35	1200			97	1260		
Age (years) mean ± SE	49.9 ± 3.4	46.5 ± 0.5	0.99	NS	52.9 ± 1.7	45.7 ± 0.5	4.04	< 0.001
Systolic BP mean ± SE (mmHg)	148 ± 4.1	143 ± 0.6	1.18	NS	149 ± 2.8	138 ± 0.8	3.96	< 0.001
Diastolic BP mean ± SE (mmHg)	92 ± 2.6	88 ± 0.4	1.17	NS	89 ± 1.5	83 ± 0.4	3.88	< 0.001
History I.H.D. (%)	11.4	7.7	*	NS	7.2	4.2	*	NS
Chest pain on effort (%)	8.6	7.1	*	NS	14.4	6.7	*	< 0.05
Possible infarction (%)	5.7	5.5	*	NS	8.2	3.6	*	< 0.05
ECG minor (%)	0	6.7	*	NS	21.6	10.0	*	< 0.01
ECG major (%)	8.6	4.5	*	NS	5.2	4.6	*	NS
Int. claudication (%)	5.6	4.4	*	NS	8.2	3.8	*	< 0.05
C.V.A. (%)	2.9	2.2	*	NS	0	1.7	*	NS

* Binomial test.

When these other risk factors were examined in relation to ECG items, irrespective of TSH, only systolic BP in females over 45 years showed an association with ECG items (MRR 2.6).

Lipids, autoimmune thyroiditis and minor degrees of hypothyroidism

There was no association between the presence of thyroid antibodies and a raised cholesterol or triglyceride or hyperlipoproteinaemia (Table 8). Females with antibodies were slightly older than those without antibodies and the mean cholesterol concentration was slightly higher (Table 8). This apparent association between antibodies and cholesterol was no longer significant when adjusted for age.

There was no association in males between an elevated TSH and hyperlipidaemia, but there was a weak association in females, which was no longer significant when adjusted for age (Table 9). These analyses were repeated after excluding all women who were pregnant, diabetic, taking oral contraceptives or other medication which might affect lipid levels. Mean lipid levels were slightly lower in the more selected samples but there was no significant association between lipid levels and thyroid antibodies or TSH.

Lipids, obesity and BP

It has been shown that the relationship between cholesterol or triglyceride and age is non-linear and thus investigation of relationships after compensating for the effect of age is difficult. Multiple linear regression analysis has been carried out but the results should be treated with caution. There appears to be a significant association between cholesterol (but not triglyceride) and obesity in males as measured by Quetelet's index, subscapular or triceps skinfold thickness. No such relationship is apparent in females although they do show an association between triglyceride and these indices. The extent of these relationships are such that a rise in Quetelet's index of 0.04 (approximately 1SD) is associated with a rise of cholesterol, in males, of about 0.25 mmol/l and of triglyceride in females of 0.15 mmol/l.

Table 8. Lipids in relation to autoimmune thyroiditis (thyroid cytoplasmic antibodies)

Variable	Males				Females			
	Thyroid antibodies		*t*	*P*	Thyroid antibodies		*t*	*P*
	Present	Absent			Present	Absent		
Number	40	1200			149	1200		
Age (years) mean ± SE	49.1 ± 2.6	46.5 ± 0.5	0.97	NS	49.7 ± 1.3	46.2 ± 0.5	2.53	< 0.05
Cholesterol (mmol/l) mean ± SE	5.9 ± 0.18	5.8 ± 0.03	0.37	NS	6.4 ± 0.12	6.2 ± 0.04	2.17	< 0.05
Triglyceride (mmol/l) mean ± SE	1.52 ± 0.13	1.46 ± 0.03	0.44	NS	1.15 ± 0.05	1.12 ± 0.02	0.65	NS
Type IIa (%) (number tested)	0 (18)	7.9 (407)	*	NS	31.0 (58)	21.1 (408)	X^2 3.01	NS
Type IV (%) (number tested)	38.9 (18)	35.4 (407)	*	NS	10.3 (58)	10.0 (408)	X^2 0.16	NS

* Binomial test.

Table 9. Lipids in relation to minor degrees of hypothyroidism (TSH ⩾ 6 mu/l)

Variables	Males				Females			
	TSH (mu/l)		*t*	*P*	TSH (mu/l)		*t*	*P*
	⩾ 6	< 6			⩾ 6	< 6		
Number	35	1200			97	1260		
Age (years) mean ± SE	49.9 ± 3.4	46.5 ± 0.5	0.99	NS	52.9 ± 1.7	45.7 ± 0.5	4.04	< 0.001
Cholesterol (mmol/l) mean ± SE	5.83 ± 0.15	5.80 ± 0.03	0.03	NS	6.55 ± 0.15	6.14 ± 0.03	2.73	< 0.01
Triglyceride (mmol/l) mean ± SE	1.56 ± 0.17	1.46 ± 0.03	0.56	NS	1.22 ± 0.06	1.12 ± 0.02	1.66	NS
Type IIa (%) (number tested)	0 (12)	7.7 (413)	*	NS	26 (50)	21.9 (415)	X^2 0.32	NS
Type IV (number tested)	25 (12)	35.8 (413)	*	NS	8 (50)	10.4 (415)	X^2 0.17	NS

* Binomial test.

This increase in Quetelet's index is also associated with a rise of 3 or 4 mmHg in systolic and diastolic BP after adjustment for age.

DISCUSSION

The confidence with which a firm diagnosis of IHD may be made is severely limited by the lack of sensitivity and specificity of the techniques available for assessing the presence of ischaemia. A firm diagnosis can be made in survivors of myocardial infarction which has been confirmed by serial ECG and enzyme changes at the time of the attack. ECG changes do not, however, persist in all patients. A history of chest pain may or may not be associated with ECG changes and Reid *et al.* (1966) in a survey of British postal workers found ECG changes in only 15% of subjects with a history of angina, in 31% of those with possible infarction and in 8% without any such history. ECG items (major and minor) were shown, in this study, to have a stronger association with a past history of IHD than symptoms of chest pain elicited by the standard questionnaire. This may be in part due to firmer evidence available to doctors who gave the diagnosis to the patient and in part to considerable observer variation in the administration of the questionnaire. The observations of Welborn *et al.* (1969) would support this view.

ECG abnormalities were coded by one observer (JGE) without reference to the questionnaire. The coding of ECG abnormalities (particularly minor S-T and T wave changes) are subject to observer variation, but the observer repeatedly checked his performance against a set of standards. Major ECG items have been shown to be relatively stable over several years whereas minor ECG items are extremely variable (Ostrander, 1966). Although it is not possible to provide direct validation of these diagnostic criteria (since no reference test is available), there is some association between major and minor ECG items and subsequent cardiac events.

The frequencies of the various criteria of IHD showed the expected increase with age. A past history of IHD was more common in men than women but the frequency of chest pain and major ECG items were similar in both sexes. Broadly similar observations were made in the community study in Busselton, Australia (Welborn *et al.*, 1969).

Lipid profiles in the Whickham population are described and although direct comparisons between surveys are not practicable unless identical laboratory techniques are used, the values are broadly similar to those observed in selected volunteers in a London factory (Lewis *et al.*, 1974). Similar mean cholesterol values were observed in Tecumseh, USA (Johnson *et al.*, 1965) but much higher cholesterol values were reported from Stockholm although triglyceride levels were similar in Whickham and Stockholm (Carlson & Linstedt, 1968). The definition of hyperlipidaemia is to some extent arbitrary but whichever criteria are chosen there is a high proportion of subjects with elevated cholesterol and triglyceride levels in the present sample.

High blood pressure is a well recognised risk factor for the development of IHD and cerebrovascular disease (Hamilton *et al.*, 1964). Evans & Rose (1971) have stressed the problems of single measurements of blood pressure in epidemiological surveys and have pointed out that a definition of hypertension is best avoided since the risk of IHD increases with blood pressure even within the normal range. The age and sex patterns seen in Whickham were similar to those seen in other Western communities.

The frequencies of the various criteria of IHD and the presumed risk factors for IHD all vary non-linearly with age. The approximate mean relative risk (MRR) of being disease

positive in the presence or absence of each factor was analysed for each age division to allow the expression of an MRR for all ages. The analysis of cholesterol, triglyceride, systolic and diastolic blood pressure, Quetelet's index (weight/height^2), skinfold thickness and smoking habits only revealed an association between systolic blood pressure in females over the age of 45 years and ECG abnormalities (major + minor). This lack of association between ECG changes and most of the factors mentioned above may be due to the low specificity of ECG criteria, but probably also reflects a defect of cross-sectional surveys. The absence of an association between IHD and smoking, obesity or cholesterol has been noted in other prevalence studies (Welborn *et al.*, 1969; Friedman *et al.*, 1966; Epstein *et al.*, 1965) and has been attributed to exclusion by death of heavy smokers and obese subjects and changes of smoking habit and weight loss in survivors of myocardial infarction. Incidence data, however, have shown an increasing risk of IHD with increasing cholesterol and triglyceride levels (Keyes *et al.*, 1963; Carlson and Bottiger, 1972; Rosenman *et al.*, 1970), obesity (Kannel *et al.*, 1967) and smoking habits (Doyle *et al.*, 1962; Doll & Hill, 1964).

There was a consistent lack of association between thyroid antibodies and IHD in males. A weak association between thyroid antibodies and chest pain on effort was noted in the female, but no association with any other more objective criterion of IHD. This lack of a consistent association between IHD and thyroid antibodies conflicts with the findings of Bastenie and his colleagues (1967, 1971) who observed an association between these variables in a hospital population and also an association between thyroiditis and cholesterol levels in females.

TSH is a highly sensitive marker of hypothyroidism (Evered *et al.*, 1973; Tunbridge *et al.*, 1977). The lack of association between high TSH and major, as opposed to minor, ECG abnormalities in females in the present study is consistent with the observations of Heinonen *et al.* (1972). These workers found no difference between mean TSH levels or thyroid antibodies in patients with heart disease or a group of carefully matched controls (although only one-third of their patients were female). The significance of the association of high TSH and minor ECG changes depends upon the interpretation of minor ECG abnormalities. If these minor changes are regarded as markers of IHD then these observations can be regarded as supporting the hypothesis that a minor degree of hypothyroidism is a risk factor for IHD. Minor ST-T wave changes do not follow the coronary heart disease distribution in women in international comparisons. Minor ECG changes, however, may be non-specific and could simply reflect myocardial depression due to hypothyroidism or some other factor rather than ischaemia. The relationship of IHD to overt myxoedema has been studied by Vanhaelst *et al.* (1967) who failed to observe any increased frequency of myocardial infarction in patients with untreated or inadequately treated thyroid failure.

The present study showed no association between cholesterol or triglyceride levels and thyroid antibodies or TSH and thus it is most unlikely that any association between IHD and minor degrees of thyroid failure could be mediated by lipid abnormalities. These observations in an epidemiological survey, therefore, directly contradict the hypothesis of Fowler *et al.* (1970). It may also be concluded that thyroid disorders make no significant contribution to the high prevalence of hyperlipidaemia in the community. The significance of the present findings can only be established by longitudinal studies which are currently being undertaken. Until or unless the incidence of established heart disease can be shown to be greater in patients with minor degrees of hypothyroidism than in otherwise comparable subjects without thyroid failure, there is no indication for the use of thyroxine in asymptomatic subjects with thyroid antibodies or a minor elevation of serum TSH.

ACKNOWLEDGMENTS

The authors thank the people of Whickham for participating in the survey; the General Practitioners of Whickham and Dunston and their receptionists for their cooperation; the staff of Whickham Cottage Hospital and Dunston Health Centre for their hospitality; the laboratory staff of the Departments of Medicine and Clinical Biochemistry, Royal Victoria Infirmary, and the Department of Haematology, Newcastle General Hospital for their collaboration; and the successive secretaries to the survey – Kay Bruder, Anna Micklethwaite, Valerie Parrish and Elke Clark – for their invaluable help. Financial support was gratefully received from the Department of Health and Social Services, the League of Friends of the Royal Victoria Infirmary, Newcastle-upon-Tyne, the Radiochemical Centre, Amersham, Mallinkrodt Ltd, Glaxo Ltd and Roche Products Ltd.

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Reprinted from

Clinica Chimica Acta, 83 (1978) 223—229
© Elsevier/North-Holland Biomedical Press

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CCA 9101

THYROID HORMONE CONCENTRATIONS IN A LARGE SCALE COMMUNITY SURVEY. EFFECT OF AGE, SEX, ILLNESS AND MEDICATION

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(Received August 10th, 1977)

Summary

Total thyroid hormone concentrations have been measured in the course of a large scale community survey to determine the distribution of these variables in the normal population and to assess the effect of age, sex, previously undetected thyroid disease and medication upon these parameters. 2779 subjects were studied. Serum T_4 concentrations were normally distributed. A progressive increase in T_4 levels with age was noted in the males, and a smaller increase in females which was concealed by the raised T_4 values secondary to oral contraceptive therapy in females under the age of 45. Serum T_3 levels were also normally distributed. There was a small reduction in T_3 with age in the males but this fall was not seen in the females. T_3 values were relatively higher in females under the age of 45 but this increase was not noted after exclusion of subjects taking an oral contraceptive. The changes in thyroid hormone concentrations with age are relatively minor (particularly with respect to T_3) in a randomly selected sample from an English town. It is suggested that the changes reported by other authors reflect the processes of selection used, and the high frequency of undetected thyroid disease, other illness and medication in hospital-based communities.

Introduction

There has been a dramatic increase in the number of diagnostic techniques available for the investigation of suspected thyroid disease over the last two decades. The relative merits and limitations of the procedures currently avail-

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able have been extensively reviewed [1—3]. The selection of an appropriate normal (reference) population to determine the diagnostic sensitivity of these procedures poses many problems. The traditional use of blood donors, hospital personnel, the relatives of patients, or "healthy" hospital patients have many practical advantages, but the use of these groups does involve processes of selection which yields subjects who are frequently unrepresentative of the general population in terms of age, sex and socio-economic group distribution. These problems are compounded, with respect to parameters of thyroid function, by the high frequency of undetected thyroid disease in the community and the effect of non-thyroidal illness and some drugs on tests of thyroid function. The objective of this paper is to examine the distribution of thyroid hormone concentrations (by direct and indirect methods) in relation to age and sex in a normal community which has been carefully screened for the presence of thyroid disease.

Materials and methods

A survey was conducted in Whickham, County Durham, a mixed urban and rural area close to Newcastle upon Tyne. Two thousand seven hundred and seventynine adults (82.4% of the available sample) were seen and their distribution by age, sex and social class has been shown to resemble closely those of the populations of Whickham and Great Britain as a whole. The survey methods have been described in detail previously [4]. Each subject was questioned and examined by a member of the survey team. Particular enquiry was made for a personal or family history of thyroid disease or other autoimmune disorder, current medication or pregnancy. Physical examination for thyroid enlargement or other signs of thyroid disease was carried out. A fasting blood sample was obtained for estimation of serum thyroxine (T_4), triiodothyronine (T_3), Thyopac-3®, thyrotrophin (TSH), and thyroid antibodies.

Serum T_4 was measured by a competitive protein binding assay (Thyopac-4®, Radiochemical Centre, Amersham). A thyroid hormone binding test (Thyopac-3®, Radiochemical Centre) was performed on all subjects. Serum T_3 was measured by radioimmunoassay [5] on a structured (with respect to age and sex) 1 in 6 subsample of patients under the age of 65 and in all those over this age and all subjects who were taking an oral contraceptive or who were pregnant at the time of the survey. Serum TSH was measured by double antibody radioimmunoassay [6] on all subjects. All sera were examined for antibodies to thyroglobulin and thyroid microsomes [7—9].

Data relating to thyroid hormone assays and thyroid hormone binding tests were analysed on a computer using the Statistical Package for the Social Sciences (SPSS) [10]. Each variable was analysed with respect to the whole population studied and in relation to age (by decade) and sex. Each variable was reanalysed after exclusion of subjects with any marker of thyroid disease (defined as a past personal or family history of thyroid disease, the presence of a goitre, a raised serum TSH (>6 mU/l) or positive thyroid antibodies), and all subjects taking drugs which were known to affect the thyroid or thyroid function tests and those who were pregnant at the time of interview. The data from the survey in relation to serum TSH and thyroid antibodies have been reported in detail elsewhere [4].

TABLE I

SERUM THYROXINE CONCENTRATIONS IN WHICKHAM (nmol/l)

Age (years)	Males					
	All subjects			Excluding any thyroid marker or medication		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	108	99.0	19.3	93	97.7	20.6
25—34	230	99.0	21.9	191	97.7	21.9
35—44	252	100.3	24.4	213	97.7	23.1
45—54	284	100.3	21.9	240	100.3	21.9
55—64	206	104.1	21.9	172	104.1	20.6
65—74	129	108.0	24.4	113	108.0	21.9
75+	57	106.7	23.1	52	108.0	21.9
All	1266	101.5	21.9	1074	101.5	21.9
Age (years)	Females					
	All subjects			Excluding any thyroid marker, medication, pregnancy or "pill"		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	126	122.1	28.3	31	109.3	28.3
25—34	318	115.7	25.7	112	110.5	24.4
35—44	268	111.8	25.7	100	105.4	21.9
45—54	239	109.3	27.0	115	109.3	23.1
55—64	258	109.3	25.7	127	110.5	24.4
65—74	173	115.7	30.8	90	115.7	20.6
75+	68	111.8	25.7	35	110.5	24.4
All	1450	113.1	27.0	611	110.5	23.1

EFFECT OF AGE ON SERUM THYROXINE CONCENTRATION

Age (years)	Males			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	99.6	22.5	97.7	21.9
45—64	101.9	23.1	101.5	21.3
65+	107.7	24.1	108.0	21.9
Age (years)	Females			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	115.7	26.3	108.0	22.9
45—64	109.3	26.3	109.8	23.8
65+	114.4	30.3	114.4	21.7

ANALYSIS

Age groups	All males	All females	Males excluding thyroid marker etc	Females excluding thyroid marker etc.
18—44 vs. 45—64	N.S.	$t = 4.16$ $p < 0.001$	$t = 2.65$ $p < 0.01$	N.S.
45—64 vs. 65+	$t = 2.94$ $p < 0.005$	$t = 2.36$ $p < 0.02$	$t = 3.22$ $p < 0.005$	N.S.
18—44 vs. 65+	$t = 4.31$ $p < 0.001$	N.S.	$t = 5.10$ $p < 0.001$	$t = 2.59$ $p < 0.02$

TABLE II

SERUM TRIIODOTHYRONINE CONCENTRATIONS IN WHICKHAM (nmol/l)

Age (years)	Males					
	All subjects			Excluding any thyroid marker or medication		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	12	1.83	0.65	10	1.74	0.66
25—34	27	1.80	0.63	20	1.75	0.61
35—44	27	1.95	0.51	23	2.00	0.48
45—54	29	1.90	0.58	24	1.89	0.54
55—64	25	1.80	0.66	19	1.57	0.41
65—74	69	1.61	0.66	54	1.61	0.64
75+	33	1.33	0.73	26	1.35	0.75
All	222	1.70	0.65	176	1.76	0.57

Age (years)	Females					
	All subjects			Excluding any thyroid marker, medication, pregnancy or "pill"		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	32	2.01	0.72	8	1.63	0.68
25—34	90	1.97	0.61	26	1.63	0.57
35—44	64	1.83	0.69	32	1.60	0.60
45—54	54	1.78	0.55	21	1.74	0.34
55—64	56	1.77	0.51	23	1.77	0.63
65—74	99	1.61	0.41	73	1.61	0.48
75+	37	1.56	0.44	28	1.58	0.55
All	432	1.78	0.68	211	1.66	0.60

EFFECT OF AGE ON SERUM TRIIODOTHYRONINE CONCENTRATION

Age (years)	Males			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	1.87	0.58	1.87	0.49
45—64	1.85	0.63	1.75	0.51
65+	1.52	0.70	1.53	0.68

Age (years)	Females			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	1.94	0.66	1.62	0.60
45—64	1.78	0.57	1.75	0.58
65+	1.60	0.51	1.60	0.50

ANALYSIS

Age groups	All males	All females	Males excluding thyroid marker etc	Females excluding thyroid marker etc.
18—44 vs. 45—64	N.S.	$t = 2.12$ $p < 0.05$	N.S.	N.S.
45—64 vs. 65+	$t = 2.92$ $p < 0.005$	$t = 2.57$ $p < 0.025$	N.S.	N.S.
18—44 vs. 65+	$t = 3.40$ $p < 0.001$	$t = 3.54$ $p < 0.001$	$t = 3.02$ $p < 0.005$	N.S.

TABLE III

THYOPAC-3 IN WHICKHAM

Age (years)	Males					
	All subjects			Excluding any thyroid marker or medication		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	109	102	6.0	94	102	6.0
25—34	231	103	6.0	192	102	6.2
35—44	254	104	7.7	214	104	7.6
45—54	287	105	6.2	243	105	5.8
55—64	208	105	6.8	173	105	6.8
65—74	130	106	6.3	114	106	6.0
75+	57	104	5.4	52	104	5.4
All	1276	104	6.7	1082	104	6.5
Age (years)	Females					
	All subjects			Excluding any thyroid marker, medication, pregnancy or "pill"		
	Number	Mean	S.D.	Number	Mean	S.D.
18—24	126	112	10.6	31	106	6.9
25—34	319	110	10.4	113	105	6.0
35—44	268	109	9.3	99	108	6.2
45—54	240	109	7.3	115	108	5.6
55—64	258	109	6.5	127	109	5.4
65—74	173	109	6.6	90	108	5.6
75+	68	108	6.2	35	107	4.9
All	1452	109	8.6	610	108	5.9

EFFECT OF AGE ON THYOPAC-3

Age (years)	Males			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	103	6.8	103	6.8
45—64	105	6.5	105	6.2
65+	105	6.0	105	5.8
Age (years)	Females			
	All subjects		Excluding thyroid marker etc.	
	Mean	S.D.	Mean	S.D.
18—44	110	10.0	106	6.2
45—64	109	6.9	109	5.5
65+	109	6.5	108	5.4

ANALYSIS

Age groups	All males	All females	Males excluding thyroid marker etc	Females excluding thyroid marker etc.
18—44 vs. 45—64	$t = 4.9$ $p < 0.001$	N.S.	$t = 4.57$ $p < 0.001$	$t = 5.58$ $p < 0.001$
45—64 vs. 65+	N.S.	N.S.	N.S.	N.S.
18—44 vs. 65+	$t = 3.58$ $p < 0.001$	N.S.	$t = 3.36$ $p < 0.001$	$t = 2.60$ $p < 0.025$

Results

Serum thyroxine concentration. Serum T_4 concentration was found to be normally distributed in the population. The mean serum T_4 was 108 (S.D. ± 25.7) nmol/l. The T_4 concentration was significantly higher in females than males (113.1 ± 27.0 compared with 101.5 ± 21.9; $p < 0.001$). Serum T_4 concentrations were found to be higher in males over the age of 65 than in men below that age. This difference was more evident when all subjects with a marker of thyroid disease were excluded from analysis and it was noted that there was a progressive rise in T_4 with age. The highest T_4 values in the females were seen in the youngest age groups studied, but these elevations could be attributed to the effects of the oral contraceptive or pregnancy (26.1% of the 18—44 age group). Exclusion of these subjects and those with any marker of thyroid disease revealed an increase in T_4 with age in the female, although this was less striking than in the male (Table I).

Serum triiodothyronine concentration. Serum T_3 concentration was found to be normally distributed in the population. The mean serum T_3 was 1.76 (S.D. ± 0.66) nmol/l. The T_3 was higher in the females than males (1.78 ± 0.68 compared with 1.70 ± 0.65 nmol/l) but this difference was not statistically significant (Table II). The serum T_3 level was found to be significantly lower in males over the age of 65 than in those below this age. Serum T_3 levels were significantly elevated in females under the age of 45 compared with women over this age, but this elevation was attributed to the oral contraceptive and pregnancy and removal of these subjects from analysis revealed no significant change in T_3 with age in the normal female.

Thyroid hormone binding test. Thyopac-3 values were normally distributed in the population. The mean value for the whole population was 107 ± 8.2. Values were significantly higher in the female than the male (109 ± 8.6 compared with 104 ± 6.7; $p < 0.001$). Lower values were noted in young males but the differences were small (although highly significant). There was no change in relation to age in the female when the whole population was studied, but further analysis after exclusion of women taking an oral contraceptive and pregnancy revealed a change with age similar to that seen in the male (Table III).

Discussion

The range of thyroid hormone levels observed during a community survey has been described. These data have been reanalysed after exclusion of all subjects with a marker of thyroid disease and drugs which are known to affect tests of thyroid function. It is clear that in the younger age groups these factors play a significant role in raising the mean serum T_4 and T_3 levels. It has been shown that there is a progressive increase in T_4 in the males with age and this is most evident after exclusion of patients on drugs and with markers of thyroid disease. There is a smaller increase in the female with age which is revealed by study of the "supernormal" population. These results conflict with earlier reports that T_4 does not change or falls slightly in relation to age (see Table IV). Serum T_3 levels have been shown to fall with age in the whole population but this fall is much less evident in the "supernormal" population and the only sig-

TABLE IV

REPORTED CHANGES IN SERUM THYROXINE AND TRIIODOTHYRONINE WITH AGE

Authors	Selection of subjects	Change in T_4 with age	Change in T_3 with age
Thomson et al. (1972) [11]	Community survey	Unchanged (PBI)	—
Snyder and Utiger (1972) [12]	"Ambulatory volunteers"	—	Reduced by 26% (males only)
Herrman et al. (1974) [13]	Not stated	Reduced by 19% (both sexes)	Reduced by 51% (both sexes)
Rubinstein et al. (1974) [14]	Not stated	Unchanged	Reduced by 21%
Hesch et al. (1976) [15]	Not stated	Reduced by 15% (both sexes)	Reduced by 31% (both sexes)
Present study	Random sample — community study	Increased by 10% (males) and 6% (females)	Reduced by 18% (males) and unchanged (females)

nificant fall is noted in males over the age of 65, compared with males under the age of 45. This fall is much smaller (18%) than those reported by other authors (see Table IV).

It is concluded, on the basis of a large scale community study, that the reported fall in thyroid hormone concentrations in relation to age has been exaggerated by selection processes. The magnitude of this fall may be amplified by failure to exclude patients taking drugs in the younger age group and by the inclusion of patients with undetected thryoid disease and non-thyroidal illness in the older age groups.

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Reprinted from the BRITISH MEDICAL JOURNAL,
24 *January* 1981, 282, 258-262

Natural history of autoimmune thyroiditis

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Abstract

One hundred and sixty-three asymptomatic people with thyroid antibodies or raised serum thyrotrophin (TSH) concentrations, or both, and 209 age-matched and sex-matched controls without either marker of thyroid disorder were followed up for four years to determine the natural history of autoimmune thyroiditis. Mildly raised TSH concentrations alone and the presence of thyroid antibodies alone did not significantly increase the risk of developing overt hypothyroidism during the four years compared with the controls. Overt hypothyroidism developed at the rate of 5% a year in women who initially had both raised TSH concentrations and thyroid antibodies.

Prophylactic treatment with thyroxine may be justified in women found to have both markers of impending

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A7622/BMJ

thyroid failure. The cost effectiveness of screening the adult population remains to be evaluated.

Introduction

Overt hypothyroidism is a common condition that develops insidiously and is often not recognised until it has been present for a considerable time. The clinical features are not specific, and the diagnosis is often made only when there is a full range of the symptoms and signs that are associated with myxoedema. In overt primary hypothyroidism serum thyrotrophin (TSH) concentrations are invariably raised, thyroxine concentrations are depressed, and thyroid antibodies are usually present, reflecting the underlying autoimmune process that is believed to be the usual cause. Raised TSH concentrations and thyroid antibodies are present before the clinical features become apparent and may be used to identify people at risk of progression to overt hypothyroidism.

The prevalence of these markers of thyroid dysfunction has been reported,[1-3] but little is known of the incidence of overt hypothyroidism in people who possess one or other or both markers. Necropsy studies of people dying from various causes have shown that lymphocytic infiltration of the thyroid (which correlates well with the presence of thyroid antibodies) is present in many such people who have lived a normal span and never had clinical evidence of thyroid disease during life.[4-6] Nevertheless, possibly an appreciable proportion of people with evidence of autoimmune thyroiditis will develop overt hypothyroidism and the earliest marker may be a raised serum TSH concentration. The present study was undertaken to determine the course of autoimmune thyroiditis and the incidence of overt hypothyroidism in the community.

Methods

The original survey was conducted in the community of Whickham near Newcastle upon Tyne.[1] All people identified in the original survey as having either thyroid antibodies or raised TSH concentrations, or both, in the absence of overt or treated thyroid disease, together with age-matched and sex-matched controls without either marker of thyroid disorder, were selected for follow-up. Participants were reassessed at two-year intervals over four years.

TABLE I—*Numbers of people selected for study and outcome of follow-up after two and four years*

	Controls			People with markers			
	Men (n = 81)	Women (n = 216)	Total(%) (n = 297)	Men (n = 61)	Women (n = 191)	Total(%) (n = 252)	Total(%) (n = 549)
			Two-year follow-up				
Seen	61	176	237 (80)	45	143	188 (75)	425 (77)
Not seen:							
Died	8	4	12 (4)	3	8	11 (4)	23 (4)
Moved	5	3	8 (3)	8	9	17 (7)	25 (5)
Refused	7	33	40 (13)	5	31	36 (14)	76 (14)
			Four-year follow-up				
Seen	51	158	209 (70)	39	124	163 (65)	372 (68)
Not seen:							
Died	2	3	5 (2)	1	2	3 (1)	8 (1)
Moved	6	6	12 (4)	1	7	8 (3)	20 (4)
Refused	2	9	11 (4)	4	10	14 (6)	25 (5)

The reassessment consisted of a symptomatic inquiry and examination particularly for evidence of thyroid disease and vascular disease. A standard questionnaire for chest pain on effort and possible myocardial infarction[7] was repeated, and blood pressure recorded using a random zero sphygmomanometer[8] with the patient recumbent after 12-lead electrocardiography. A fasting blood sample was taken for the following measurements: serum concentrations of TSH,[9] thyroxine (Thyopac-4, Amersham), and triiodothyronine[10]; triiodothyronine Sephadex uptake (Thyopac-3, Amersham) used in conjunction with the thyroxine concentration to derive a free thyroxine index,[11] thyroglobulin antibodies,[12] and thyroid cytoplasmic (microsomal) antibodies by immunofluorescence[13] and microsomal haemagglutination techniques[14]; antibodies to gastric parietal cells, smooth muscle, mitochondria, and antinuclear factor measured by an immunofluorescence technique; and serum concentrations of cholesterol (Technicon method file N24a), triglyceride,[15] and glucose (oxidase method, Autoanalyser).

Serum from each subject was divided, half being used immediately for the above investigations and the remainder stored at $-20^{\circ}C$ until the end of the four-year study. Basal samples and those obtained at two-year and four-year follow-up were then retested in the same assay.

Thyroglobulin antibodies were scored as present at a titre of 1/20 or more and microsomal antibodies at a titre of 1/100 or more; serum TSH concentrations were defined as raised when greater than 6 mU/l in the original survey.

A postal questionnaire was sent after four years to all the other participants in the original Whickham survey, who had not been selected for special follow-up as above. This questionnaire asked if the person had developed any thyroid, heart, or blood pressure troubles since seen in the original survey. Affirmative replies were checked against family practitioner and hospital records. Practitioner records were also checked for those who failed to reply. Certified causes of death were ascertained for all people who had died during the follow-up. Data from completed questionnaires were transferred to punch cards, which were then processed using the Statistical Package for the Social Sciences.[16]

Results

Two hundred and fifty-two people with thyroid antibodies or raised TSH concentrations, or both, and 297 age-matched and sex-matched controls without either marker of thyroid disorder were selected for the follow-up study. Patients with one or both markers who had received antithyroid treatment were not included in the study. The proportion of women to men was roughly 3:1, reflecting the predominance of autoimmune thyroiditis in the former. The mean ($\pm$SD) age at selection for all subjects studied was 49 ± 15 years.

Three-quarters of those selected were seen for reassessment after two years and two-thirds after four years. At four years the proportions

of subjects who had died (5·6%), moved out of the area (8·2%), or refused to participate (18·4%) were similar in the subjects with markers and controls (table I). The age and sex distributions of the 372 people remaining in the study at the end of the four years were similar in the controls and people with markers. The marker group was subdivided into: (*a*) those with antibodies and normal initial TSH concentrations; (*b*) those with raised initial TSH concentrations but no antibodies; and (*c*) those with both antibodies and raised initial TSH concentrations (table II).

TABLE II—*Numbers of people who completed four years of follow-up*

	Men	Women
Controls	51	158
People with markers:	39	124
Antibodies alone	20	67
Raised TSH alone	9	27
Antibodies and raised TSH	10	30
	90	282

The postal questionnaire was sent to 2163 people without raised TSH concentrations or thyroid antibodies seen in the original survey, and to 76 people who had declined to participate in the special follow-up study. Replies were received from 1748 (78%), and information on the remaining 491 (22%) was sought from practitioners' records; 142 (6%) had died and 205 (9%) had moved from the area.

Causes of death for all subjects followed up in any way were ascertained from death certificates and were cardiovascular disease (2·7%), cerebrovascular disease (1·6%), and miscellaneous, including malignant disease (2·2%). The proportions of deaths due to these causes were not significantly different between the controls and the people with markers or those sent the questionnaire.

CHANGE IN THYROID FUNCTION

Antibodies

Sera were available from all but four of the 372 people seen throughout the four-year follow-up. Thirty men and 97 women were initially positive for thyroid (predominantly microsomal) antibodies. Nine subjects were initially weakly positive for antibodies (five thyroglobulin and four microsomal); this was not confirmed on retesting the stored sera, and the results remained negative at two-year and four-year follow-up. These nine may be regarded as originally giving false-positive results.

Three women were strongly positive for microsomal antibodies

TABLE III—*Mean TSH concentrations (mU/l) basally and at two-year and four-year follow-up, comparing values obtained originally and when samples were reassayed at end of study**

	No of subjects	Original values			Values on reassay		
		Basal	2-year	4-year	Basal	2-year	4-year
		Men					
Controls	49	2·0	1·5	2·6	1·6	1·5	1·2
People with markers:							
Antibodies alone	17	2·8	3·0	4·5	3·2	2·8	2·8
Raised TSH alone	9	8·1	2·7	5·3	4·0	2·9	3·4
Antibodies and raised TSH	7	8·4	5·9	11·1	5·8	5·9	9·2
		Women					
Controls	129	2·5	2·1	3·6	1·9	1·8	1·8
People with markers:							
Antibodies alone	53	2·7	2·4	4·4	2·4	2·4	2·8
Raised TSH alone	27	7·4	2·7	5·0	3·4	3·1	3·2
Antibodies and raised TSH	21	11·0	8·5	12·8	7·0	8·0	8·6

*Subjects who became overtly hypothyroid and were treated during the study and those for whom samples were missing are excluded.

TABLE IV—*Mean free thyroxine index basally and at two-year and four-year follow-up**

	Men				Women			
	No of subjects	Free thyroxine index			No of subjects	Free thyroxine index		
		Basal	2-year	4-year		Basal	2-year	4-year
Controls	51	94	112	97	127	101	112	103
People with markers:								
Antibodies alone	20	97	104	96	53	100	103	96
Raised TSH alone	9	100	114	88	20	89	110	96
Antibodies and raised TSH	10	79	90	82	20	84	76	71

*Subjects who became overtly hypothyroid and were treated during the study and those for whom samples were missing are excluded.

(one of whom was also strongly positive for thyroglobulin antibodies), and this was confirmed on retesting the original sera, but antibodies were absent at two-year and four-year follow-up. Antibodies thus disappeared from three out of 88 women over four years.

Sixty men (51 controls and nine subjects with raised initial TSH concentrations) and 185 women (158 controls and 27 subjects with raised initial TSH concentrations) had no antibodies initially. Ten women who were thought to have no antibodies when first tested but were positive for microsomal antibodies after two and four years were found to have been positive for antibodies when the original sera were retested; they may be regarded as originally giving false-negative results. Antibodies developed in 16 women (13 controls and three subjects with raised initial TSH concentrations) who had no antibodies originally, confirmed on retesting. Antibodies thus appeared in 16 out of 175 women over four years. The antibody state of the men did not change throughout the study.

TSH

Sera for assay of TSH concentrations were available from 353 of the 372 people seen throughout the follow-up. Samples were obtained from 190 controls (139 women and 51 men) and 163 subjects with markers (124 women and 39 men). TSH concentrations in all the groups studied were significantly lower at the two-year follow-up than basally and at four-year follow-up. This interassay variation was eliminated when all the avilable sera from each subject were retested in the same assay at the end of the follow-up, although the reassay values tended to be lower than the original values (table III).

Mean TSH concentrations in the controls did not vary significantly over the four years when retested, and only 5% of the women and none of the men developed a concentration above 6 mU/l. Mean concentrations in the 67 women and 20 men who initially had only antibodies as a marker were significantly higher than those in the controls throughout the study but remained in the normal range, and only 10 (12%) of these subjects developed a concentration above 6 mU/l over the four years. Mean concentrations in the 27 women and nine men who initially had raised concentrations but no antibodies tended to regress to the mean over four years, although this decline was not significant when the samples were reassayed. The concentrations in this group ranged from 6 to 10 mU/l initially and rose above this subsequently in only one subject. Mean concentrations in the 30 women and 10 men who initially had antibodies and raised concentrations were significantly higher than those in the people who initially had only one marker. Concentrations remained raised in this group throughout the study (excluding those who developed overt hypothyroidism, whose concentration fell in response to treatment—see below). The range of initial concentrations in this group was 6-24 mU/l; the concentration fell in only one subject, in whom it had initially been about 6 mU/l.

Circulating thyroid hormone concentrations

Mean free thyroxine index was lowest at all stages in the people with both markers (table IV). Mean values were higher in all subjects at two-year follow-up than originally or at the four-year follow-up, except in women with both markers, in whom the index fell steadily. The fall in the index in this group was significant, whereas fluctuations in the controls and other marker groups may be explained by normal interassay variation.

Mean triiodothyronine concentrations were not significantly different between controls and any marker group either initially or at two- and four-year follow-up.

Thyrotoxicosis

Thyrotoxicosis developed in two women, both controls, who developed clinical as well as biochemical features of hyperthyroidism. Five other women, who did not have clinical evidence of hyperthyroidism, showed borderline increases in serum thyroxine concentrations and free thyroxine index, which were not confirmed on retesting.

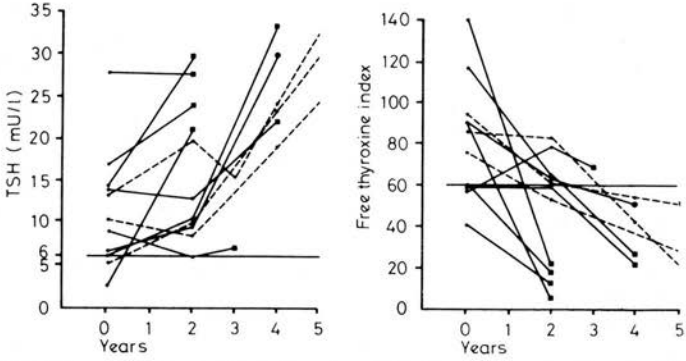
DEVELOPMENT OF OVERT HYPOTHYROIDISM

Seven women and one man became overtly hypothyroid during the study, four in the first and four in the second two years. All eight had thyroid antibodies and seven raised TSH concentrations when first seen. All developed symptoms and signs consistent with clinical hypothyroidism. Their TSH concentrations rose and thyroxine concentrations fell before treatment with thyroxine (figure), which led to clinical improvement and return of these two concentrations to the normal range. Three other women, who had antibodies and raised TSH concentrations initially (6, 10, and 14 mU/l respectively), also showed a progressive rise in TSH concentrations and fall in thyroxine concentrations to below the normal range but were not recognised as clinically hypothyroid at the four-year follow-up. It was predicted that they would soon become overtly hypothyroid, and this occurred in all three within the following year. These three subjects although biochemically hypothyroid were not included in the subsequent analysis of the incidence of overt hypothyroidism.

One woman of the 67 who initially had antibodies but normal TSH concentrations became overtly hypothyroid during the four years. None of the controls and none of the subjects who initially had raised TSH concentrations but no antibodies became overtly hypothyroid.

Six of the 30 women and one of the 10 men who initially had both raised TSH concentrations and thyroid antibodies became overtly hypothyroid during four years.

None of the subjects sent the questionnaire after four years had developed overt thyroid disease; thus, on the assumption that those identified in the group with markers represent all the cases of overt



TSH concentrations and free thyroxine index in seven women (——■) and one man (——●) who developed overt hypothyroidism during the study and three women (- - - -) who subsequently developed clinical as well as biochemical evidence of hypothyroidism.

hypothyroidism occurring in the original sample of the population (1285 men, 1494 women) the annual incidence of overt hypothyroidism is between 1 and 2/1000 women and about 1/5000 men.

ELECTROCARDIOGRAPHIC CHANGES

An association between minor electrocardiographic changes and raised TSH concentrations but not the presence of antibodies was found in women in the original survey. This association was present in women selected for follow-up but disappeared over the four years. Fifteen women initially had raised TSH concentrations and minor electrocardiographic changes, but both of these returned to normal in six (four of whom were treated with thyroxine); the TSH concentration fell spontaneously to normal in six others, whose electrocardiographic abnormalities persisted (four of whom had evidence of ischaemic heart disease); and the concentrations remained raised in three, whose electrocardiograms returned to normal.

LIPID CONCENTRATIONS

Mean fasting cholesterol and triglyceride concentrations were not significantly different between controls and the marker group at all stages of follow-up. Mean ($\pm$SD) basal cholesterol concentrations in the women with both markers of thyroid disease were higher than those in the controls ($6{\cdot}9 \pm 1{\cdot}20$ *v* $6{\cdot}3 \pm 1{\cdot}14$ mmol/l; 267 ± 46 *v* 244 ± 44 mg/100 ml), and this difference persisted but did not increase during the

follow-up (6·7 ±1·24 *v* 6·1 ±1·19 mmol/l (259 ±48 *v* 236 ±46 mg/100 ml) after four years). Mean cholesterol concentrations in the women with only one marker were not significantly different from those in the controls at any stage. Among the eight people who developed overt hypothyroidism four originally had cholesterol concentrations below and four above the mean in the controls.

Discussion

By itself a raised TSH concentration did not appear to have any predictive value for the development of overt hypothyroidism over four years. Possibly this was because most of the concentrations were only slightly increased—that is, between 6 and 10 mU/l. The mean and range of TSH concentrations were considerably higher in subjects in whom TSH concentrations were raised and antibodies present: 5% of these women developed overt hypothyroidism a year. In a highly selected group of subjects with autoimmune thyroiditis whose initial TSH concentrations were above 19 mU/l Gordin and Lamberg[17] showed that overt hypothyroidism developed at a rate of roughly 10% per annum over three years. Thus the higher the TSH concentration the greater the likelihood of developing overt hypothyroidism. We were unable to show any relation between the degree of increase of TSH concentrations and increasing titre of antibodies.

Interassay variation is considerable in all radioimmunoassays and was a major handicap in our evaluation of borderline values, most of which fell at two years but were higher at four years. Repeat assays of all the stored samples after four years eliminated interassay variation but did not exclude the effect of deterioration of the earlier samples because of longer storage. All samples were taken between 9 and 11 am so that diurnal variation was unlikely to account for the observed variation, and people were seen close to the anniversary of their original visit, so that seasonal variation was not a factor. Nevertheless, the mild fluctuations in TSH values might have been spontaneous, while the mean free thyroxine index was higher at two years than basally or at four years. In practical terms, in the absence of any evidence of underlying autoimmune thyroiditis mildly raised TSH concentrations are of doubtful clinical importance and should be rechecked.

Antibodies were initially scored as present at very low titres—that is, 1/20 for thyroglobulin, 1/10 for cytoplasmic immunofluorescence, and 1/100 for microsomal haemagglutination. Although microsomal haemagglutination occurred in over 80% of subjects in whom results of immunofluorescence testing

were positive,[14] perhaps not surprisingly up to 5% of tests yielded false-positive or false-negative results. Thyroglobulin titres of 1/40 and microsomal titres of 1/200 or more disappeared genuinely from only three subjects. It was to be expected that antibodies would appear spontaneously in some women in the middle age range, and our annual incidence of 2% is consistent with the known rapid rise in the prevalence of antibodies in women aged over 45. Mean microsomal antibody titres tended to increase (by one dilution—that is, from 1/400 to 1/800) over the four years in those in whom these antibodies were present at the beginning of the study.

The meaning of minor electrocardiographic changes remains open to question. Minor ST and T-wave changes have some prognostic importance for ischaemic heart disease[18 19] but are evanescent. In the original survey[20] minor electrocardiographic changes showed a weak association with raised TSH concentrations, but this disappeared during follow-up. The proportion of deaths due to cardiovascular disease was not significantly different between controls and the marker groups. Thus there was no evidence of any association between ischaemic heart disease and symptomless autoimmune thyroiditis,[21] but the numbers were small.

Mean fasting cholesterol and triglyceride concentrations were not significantly different between controls and subjects with only one marker. Although mean cholesterol concentrations were slightly higher in women with both markers, they were within the normal range, did not change during follow-up, and had no prognostic value in identifying those who developed overt hypothyroidism. There was thus no evidence to support the hypothesis that raised cholesterol concentrations indicate premyxoedema.[22 23]

PREVENTION OF OVERT HYPOTHYROIDISM

In our survey three women with unequivocal biochemical hypothyroidism were not detected clinically. Should people with antibodies and raised TSH concentrations be treated with thyroxine to prevent the development of overt hypothyroidism? The people in this study were not patients but randomly selected from a cross-section of the community. Before advocating widespread screening of adult women for raised TSH concentration and antibodies it is important to decide whether subjects having these should be treated. Arguments in favour of treatment are as follows: (*a*) overt hypothyroidism develops insidiously and may impair mental and physical function; (*b*) the incidence of overt hypothyroidism in women with both markers is 5% per annum. Moreover, this is cumulative, so that after four years 20% are affected and after 10 years half might have overt hypothyroidism; (*c*) treatment is simple, cheap, and effective; and (*d*) women may not realise they were functioning

below normal until they realise how much better they feel with treatment.

Arguments against treatment of asymptomatic individuals with both markers include: (*a*) many women live their normal span without ever developing clinical evidence of thyroid disorder; (*b*) treatment may prevent one in 20 developing overt hypothyroidism in one year, but 19 will be treated unnecessarily; (*c*) thyroxine may be dangerous particularly in exacerbating ischaemic heart disease; (*d*) some of the cases of overt hypothyroidism will be recognised anyway; and (*e*) some people with overt hypothyroidism fail to take their thyroxine regularly, and hence probably those women who are asymptomatic and feel no benefit from treatment may default.

If the data from Whickham are applicable to the population of Great Britain with roughly 20 million adult women, 5% (that is, one million) would have both raised TSH concentrations and thyroid antibodies. Of them, 5% per annum (that is, 50 000) might develop or be prevented from developing overt hypothyroidism. The logistics of setting up a screening programme for all adult women would be considerable. Higher returns for the investment would be obtained from screening, say, only women over the age of 45. The cost effectiveness of such a programme would depend on the balance between its costs and the cost of the morbidity of untreated hypothyroidism, which is much more difficult to determine.

Until now we have not advocated treatment for *asymptomatic* people found to have thyroid antibodies or raised TSH concentrations on the grounds that the natural course was not known and that the benefits of treatment might not outweigh the disadvantages. Now our view has been modified by the findings of the present study. It seems reasonable to recommend thyroxine treatment for women presenting to their doctor who are found to have both raised TSH concentrations and antibodies (but neither alone) provided there is no hazard—for example, exacerbating ischaemic heart disease. Nevertheless, we are not advocating a widespread screening programme for autoimmune thyroditis in the whole population: this must await a precise economic evaluation. On the other hand, it may be worth while screening high-risk groups such as postmenopausal women attending a clinic for any reason or in hospital wards (particularly geriatric) and women attending diabetic clinics or thyroid clinics who have had destructive treatment of the thyroid by surgery or radioiodine.

We thank the people of Whickham for participating in the study; the general practitioners of Whickham and Dunston and their staff for their co-operation; the staff of Whickham Cottage Hospital and

Dunston Health Centre for their hospitality; and the secretarial and laboratory staff of the departments of medicine and clinical biochemistry, Royal Victoria Infirmary, and the department of haematology, Newcastle General Hospital, for their collaboration.

Financial support was gratefully received from the Department of Health and Social Security; the League of Friends of the Royal Victoria Infirmary, Newcastle upon Tyne; the Radiochemical Centre, Amersham; Mallinkrodt Limited; Glaxo Limited; and Roche Products Limited.

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(*Accepted 6 November 1980*)

Printed in Great Britain by Bourne Offset (Iver) Ltd., Iver, Bucks.

PAPERS AND SHORT REPORTS

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Intensive attention improves glycaemic control in insulin-dependent diabetes without further advantage from home blood glucose monitoring: results of a controlled trial

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Abstract

Forty-six diabetics treated with twice-daily insulin were seen every two weeks for six months in an intensive education programme aided by regular home urine glucose testing. Control was improved with a decrease in 24-hour urinary glucose excretion (median 138 mmol/24 h (24·8 g/24 h) falling to 70 mmol/24 h (12·6 g/24 h); $p < 0.002$), glycosylated haemoglobin concentration (mean 11·4 ± SD 2·3% falling to 10·4 ± 1·5%; $p < 0.001$), and Diastix score (median 3·0 falling to 1·3; $p < 0.001$). There was no reported increase in hypoglycaemia.

Thirty-eight of the diabetics proceeded to a nine-month randomised cross-over study of the effect on blood glucose control of monitoring urinary glucose or blood glucose measured visually or by a reflectance meter using appropriate reagent strips. No further improvement in control was observed after home blood glucose monitoring. Nevertheless, 29 out of 37 patients preferred blood to urine glucose monitoring.

During both the education and cross-over studies there was evidence of an initial improvement in control followed by deterioration. This was independent of the monitoring method used in the cross-over period and may have been due to waning enthusiasm.

Despite patient enthusiasm and other reports to the contrary, home blood glucose monitoring offered no improvement in control over intensive attention and conventional urine glucose monitoring.

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Introduction

Other studies have shown how poorly many diabetics understand their disease and its management[1–3] and how difficult it may be to obtain information and help from medical and nursing staff and dietitians.[4–7] Attempts to improve patient education and access to advice have been partially successful[3 4 7 8] but metabolic control as assessed by blood and urine glucose estimations remained unchanged.[8] A major problem is the inadequacy of the methods used to assess diabetic control, investigators relying at best on random blood or urine glucose estimations,[8] with their inherent drawbacks,[9 10] or even the frequency of admission to hospital.[1 7] Glycosylated haemoglobin assays, however, now permit the collection of more accurate information about glycaemic control.

Increasing awareness of the importance of good control of blood glucose values in diabetes[11–13] and acknowledgment of the limitations of urine testing[9 10] have led to the introduction of self-monitoring of blood glucose concentrations by the patient as a potential method of improving control.[14–19] The improved control attributed to home blood glucose monitoring in these reports has not been analysed to separate the effect of the inevitable increased attention by the patient and staff from the effect of monitoring alone.

To try to overcome this problem we studied a group of patients who took part in a six-month period of optimisation of diabetic control which included frequent clinic visits and regular conventional urine testing. This was followed by a nine-month

period when patients monitored and managed their own progress using home blood glucose or urine glucose estimations in a cross-over study. To investigate the importance of a reflectance meter as opposed to visual assessment of glucose reagent strips we included both procedures in the second part of the study. Throughout the 15 months objective measurements of diabetic control were recorded.

Patients and methods

A total of 154 patients on our diabetic clinic register aged 16-60 years and receiving twice-daily insulin were invited by letter to participate in a study of diabetic control. They were told that the study would include intensive efforts to improve their control, firstly with conventional urine testing and subsequently with home blood glucose monitoring as aids. Pregnant patients, those taking drugs known to alter carbohydrate metabolism (for example, oral contraceptives), and patients with renal disease (plasma creatinine concentration >200 μmol/l (>2·26 mg/100 ml)) or proliferative retinopathy were excluded.

Table I shows the design of the study. During the initial six-month optimisation period patients managed their diabetes solely with regular urine tests. They were then allocated at random to one of six groups to define the sequence of the subsequent monitoring methods. Monitoring of urine with glucose reagent strips continued throughout the nine-month cross-over period but was the only method used in the periods marked "urine." In the periods marked "visual" and "meter" self-monitoring of blood glucose values was performed in addition using, respectively, glucose reagent strips read by eye against a colour scale and strips read in a reflectance meter.

Fifty-four patients entered the study but eight were subsequently excluded (table II). Results are presented for 27 men and 19 women completing the six-month optimisation period (mean % ideal body weight[20] 104±SD 10, range 83-131; mean age 34±SD 9 years, range 16-51).

Two patients excluded from the initial six-month analysis rejoined at the beginning of the cross-over study (the patient taking once-daily insulin had returned to twice-daily injections, and one pregnant diabetic had a therapeutic abortion on social grounds, but she then withdrew again soon after rejoining). Thus 48 patients proceeded to the cross-over period but 10 were excluded (table II). Results are presented for the 38 patients completing this phase (24 male, 14 female; mean % ideal body weight 103±9, range 85-134; mean age 36±9 years, range 17-51). All patients began the cross-over study at the same time. Hence a few had less than 12 visits in the optimisation period because occasional visits were made at three-weekly intervals owing to other commitments. Thus all 46 patients completed nine visits, 45 completed 10, 44 completed 11, and 37 completed 12 visits before entering the cross-over period.

TABLE I—*Study design (method of monitoring diabetic control)*

Optimisation period 6 months	Cross-over period			
	3 months	3 months	3 months	No of patients*
Urine	Urine	Visual	Meter	7
Urine	Urine	Meter	Visual	6
Urine	Visual	Urine	Meter	6
Urine	Visual	Meter	Urine	7
Urine	Meter	Urine	Visual	6
Urine	Meter	Visual	Urine	6

Urine = Semiquantitative urine tests (Diastix). Visual = BM-Glycemie 20-800 strips read visually. Meter = Reflotest-Glucose strips read with a reflectance meter.
*Number of patients shown for each subgroup in cross-over period.

TABLE II—*Reasons for withdrawal from study*

No entering optimisation period		54
Failure to return to clinic	4	
Pregnancy	2	
Moved from area	1	
Period on once-daily insulin	1	
No completing optimisation period		46
No entering cross-over period		48*
Pregnancy	3	
"Insufficient time"	2	
"Social problems"	2	
Non-compliance	2	
"Nerves"	1	
No completing cross-over period		38

*Two patients re-entered at beginning of cross-over period (see text).

PROTOCOL

Patients were seen fortnightly by either RW or PDH with 30 minutes available for each visit. Between visits patients were encouraged to telephone with problems, but they rarely did this. An initial assessment of dietary intake was obtained by an experienced dietitian, who was available for consultation throughout. At each visit patients were weighed, symptoms assessed, and a record made of current dietary intake and insulin dose. The number of symptomatic hypoglycaemic episodes in the preceding fortnight was noted (defined as any event necessitating additional carbohydrate to correct symptoms thought by the patient to be due to hypoglycaemia).

Patients were instructed on the importance of a regular carbohydrate intake and on modification to diet and insulin dosage, depending on changes in weight, physical activity, or diabetic control as assessed by home tests. Patients were instructed carefully in the performance and interpretation of urine and blood tests, and this was monitored frequently throughout the study. They were told to take their insulin 20-30 minutes before meals, and injection techniques and sites were periodically assessed. All subjects continued with twice-daily short-acting (Actrapid (Novo) or soluble (Wellcome)) and intermediate-acting insulins (Insulatard (Nordisk), isophane (Wellcome), or Monotard (Novo)).

Urine tests were performed by the patients using semiquantitative urine tests (Diastix, Ames) four times daily, before main meals and at bedtime. Results were recorded graphically. For later statistical analysis Diastix results were scored by the medical staff (0% = 1; 0·1% = 2; 0·25% = 3; 0·5% = 4; 1% = 5; 2% = 6) and median scores for each two-week period thus obtained.

Visual estimations of blood glucose concentrations were performed by patients using BM-Glycemie 20-800 reagent strips (Boehringer Mannheim).[21–24] A series of Glucochek meters (Medistron Ltd, Alpine Works, Crawley, Sussex) were modified to read Reflotest-Glucose reagent strips (Boehringer Mannheim) rather than Dextrostix (Ames), in view of the better overall performance characteristics of the former.[23] Estimations below 4 mmol/l (72 mg/100 ml) on the meter were repeated with visual reading of the Reflotest-Hypoglycemie reagent strip (Boehringer Mannheim) because the standard Reflotest-Glucose strips were not accurate at hypoglycaemic concentrations. The accuracy of the two systems within the first fortnight was assessed for each patient by taking simultaneous samples for laboratory analysis of blood glucose values and comparing patient and laboratory results. Correlation coefficients for visually read samples (n = 37) and meter-read samples (n = 35) were 0·79 ($p < 0·001$) and 0·78 ($p < 0·001$), respectively.

Visual readings were overestimated as often (17 times) and by the same amount (median error 1·4 mmol/l (25 mg/100 ml), range 0·2-7·0 mmol/l (4-126 mg/100 ml)) as they were underestimated (20 times, median error 1·4 mmol/l (25 mg/100 ml), range 0·1-8·1 mmol/l (2-146 mg/100 ml)). Meter-read samples, however, were overestimated (27 times, median error 2·2 mmol/l (40 mg/100 ml), range 0·1-8·0 mmol/l (2-144 mg/100 ml)) more than they were underestimated (eight times, median error 0·5 mmol/l (9 mg/100 ml), range 0·4-1·4 mmol/l (7-25 mg/100 ml)). Patients were asked to monitor their blood glucose values on at least two days each week with samples taken before and one hour after each main meal and before bedtime. They were also encouraged to perform intermittent estimations on intervening days, concentrating particularly on times known to be associated with problems with control. Sampling was aided using atraumatic lancets (Monolet, Sherwood) and a spring-loaded capillary puncture device (Autolet, Owen-Mumford). Advice on adjusting insulin dosage from results obtained was similar to the guidelines advocated by Skyler *et al*,[25] with adjustment of insulin dosage if a clear pattern arose in successive profiles. Compensation for variations in activity was usually dietary except for strenuous exercise, when a reduction in insulin dosage was also advised.

One or two days before each visit patients were asked to obtain a seven-point capillary blood glucose profile consisting of samples collected before and one hour after meals and before bedtime. Blood was collected into fluoride-oxalate containers for laboratory analysis. A single 24-hour urine collection was also obtained before each visit for subsequent glucose analysis.

At every clinic attendance venous blood samples were obtained for glycosylated haemoglobin estimation. At the beginning and end of

the optimisation period and at the end of each cross-over period blood (after a meal) was collected for analysis of serum total cholesterol and plasma urea and creatinine concentrations. Postprandial blood samples for plasma C-peptide estimation were obtained on one to three (median 3) occasions from each subject. C-peptide results were expressed as positive or negative.

At the first visit in the optimisation period patients were instructed in the collection of samples, and thus the first results for median Diastix and blood and urinary glucose measurements were obtained at visit 2.

Three months after the cross-over study patients completed a questionnaire asking about attitudes to the various methods of monitoring control.

CHEMICAL METHODS

Whole blood glucose was analysed by a standard automated glucose oxidase assay (Technicon AA II, Boehringer GOD-PAP). Urine glucose was estimated by a hexokinase method (Roche) with aqueous chlorhexidine (Hibitane, 20% vol/vol) as preservative (one volume to 100-400 volumes of urine). Glycosylated haemoglobin was analysed by a modification of the colorimetric method of Fluckiger *et al.*[26 27] Interassay variation for the glycosylated haemoglobin estimation was 3·8% and the reference range 5·0-8·2%. Autoanalyser techniques were used for estimating serum total cholesterol (Technicon AA II, fully enzymatic GOD-PAP) and plasma urea and creatinine concentrations (Technicon SMA 6/60). Plasma C-peptide was estimated with antibody M1230, which has a detection limit of 0·06 nmol/l.[28]

STATISTICAL METHODS

Statistical analyses were performed on an IBM computer (370/168) using programs from the Statistical Package for Social Sciences. Differences from baseline measurements were analysed with Student's paired *t* test (glycosylated haemoglobin, pre- and postprandial, and mean blood glucose, serum cholesterol, plasma urea and creatinine, dietary carbohydrate intake, insulin dose, and body weight) or Wilcoxon's matched pairs signed-rank test (24-hour urinary glucose excretion, frequency of hypoglycaemia, and Diastix scores). Differences between groups of patients at individual times were assessed by Student's unpaired *t* test or Wilcoxon's unpaired rank test. Correlations were sought by linear regression analysis or Spearman's ranking method as appropriate, and trends of change over the study period sought by analysis of variance or the Kruskal-Wallis test for differences between visits.

Values are expressed as the mean, mean ± SD, or median + range.

A mean preprandial blood glucose value was calculated for each individual blood glucose profile only if three or more capillary blood samples were provided (out of a possible four). Similarly, a mean overall blood glucose value (preprandial and postprandial samples) was determined only if six or more samples were obtained (out of a possible seven). For the group as a whole mean blood glucose represents the mean ± SD of the mean blood glucose concentrations in all subjects with a valid profile at a particular visit.

Results

OPTIMISATION PERIOD (SIX MONTHS)

24-Hour urinary glucose excretion

Urinary glucose excretion fell during the study (table III). Initial median 24-hour values (138 (range 1-591) mmol/24 h (24·8 (range 0·2 106·4) g/24 h) at visit 2) fell significantly using paired comparisons by visit 10 (70 (1-508) mmol/24 h (12·6 (0·2-91·4) g/24 h); $p < 0·002$) and visit 11 (52 (1-745) mmol/24 h (9·4 (0·2-134·1) g/24 h); $p < 0·02$). The correlation between median 24-hour urinary glucose excretion and visit number was significant ($n = 11$; $r_s = -0·79$; $p < 0·01$). Figure 1 shows the median change in median 24-hour urinary glucose excretion over the six months.

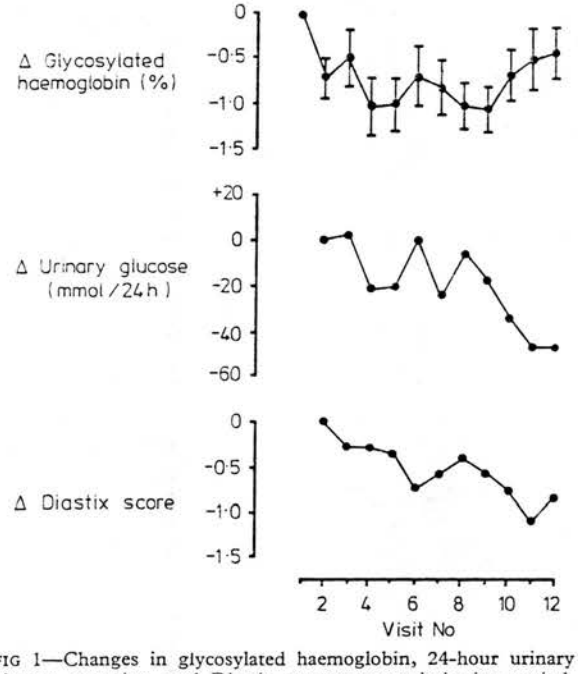

FIG 1—Changes in glycosylated haemoglobin, 24-hour urinary glucose excretion, and Diastix score over optimisation period. Results expressed as mean change (± SEM) in mean glycosylated haemoglobin, median change in median 24-hour urinary glucose, and median change in median Diastix score. Visits made two-weekly.

Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h.

TABLE III—*Twenty-four-hour urinary glucose excretion, Diastix scores, and glycosylated haemoglobin and mean blood glucose concentrations during initial six-month optimisation period*

Visit No	24-Hour urinary glucose (mmol/24 h)				Diastix score				Glycosylated haemoglobin (%)			Mean blood glucose (mmol/l)			
												Preprandial		Overall§	
	n	Median	Range	p†	n	Median	Range	p†	n	Mean ± SD	p‡	n	Mean ± SD	n	Mean ± SD
1	*	*	*	*	*	*	*	*	46	11·4 ± 2·3	—	*	*	*	*
2	45	138	1-591	—	45	3·0	1-6	—	44	10·7 ± 2·0	<0·01	26	8·1 ± 3·7	9	10·3 ± 4·1
3	45	108	1-934	NS	46	2·7	1-6	<0·05	45	11·0 ± 2·0	NS	33	8·6 ± 2·8	13	8·7 ± 2·7
4	42	82	2-823	NS	46	2·1	1-6	<0·02	46	10·4 ± 1·8	<0·005	33	8·7 ± 2·6	18	8·9 ± 2·0
5	43	95	1-747	NS	45	2·3	1-6	<0·05	41	10·4 ± 1·9	<0·005	39	9·0 ± 3·4	23	9·4 ± 3·2
6	43	106	3-659	NS	45	1·7	1-5	<0·001	34	10·8 ± 1·5	NS	41	8·8 ± 3·4	27	9·4 ± 3·3
7	44	88	2-959	NS	45	1·6	1-6	<0·002	40	10·7 ± 2·2	<0·02	43	8·4 ± 2·7	26	8·7 ± 2·5
8	42	118	1-624	NS	45	1·8	1-6	<0·002	46	10·4 ± 1·6	<0·001	44	8·6 ± 3·2	30	9·2 ± 2·4
9	44	65	1-937	NS	46	1·6	1-5	<0·001	46	10·4 ± 1·5	<0·001	41	7·7 ± 2·9	21	8·7 ± 3·4
10	43	70	1-508	<0·002	43	1·4	1-5	<0·001	45	10·7 ± 1·9	<0·05	39	9·2 ± 3·7	27	10·6 ± 4·1
11	40	52	1-745	<0·02	42	1·3	1-5	<0·001	44	10·9 ± 1·8	NS	40	8·2 ± 2·9	27	9·1 ± 3·3
12	33	46	1-685	NS	37	1·7	1-5	<0·001	37	11·0 ± 1·9	NS	28	8·5 ± 4·6	17	10·2 ± 4·7

*Patients instructed on sampling at visit 1 and therefore results available from visit 2 only. Visits made two-weekly.
†Differences from visit 2 tested for significance with Wilcoxon's matched pairs signed-rank test.
‡Differences from visit 1 tested for significance with Student's paired *t* test.
§Mean overall blood glucose includes preprandial and postprandial samples. By Student's paired *t* test no mean blood glucose value significantly different from visit 2.
Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h. Blood glucose: 1 mmol/l ≈ 18 mg/100 ml.

Diastix score

Median Diastix scores fell over the study period ($p < 0.001$ for the last four visits) (table III). Paired testing against visit 2 was significant at all subsequent times, and the correlation between Diastix scores and visit number was highly significant ($r_s = -0.82$; $p < 0.005$). This trend was confirmed by the Kruskal-Wallis test ($p < 0.005$). Figure 1 shows the median change in Diastix score during the study.

Glycosylated haemoglobin

During the education period glycosylated haemoglobin concentrations fell rapidly from visit 1 ($11.4 \pm 2.3\%$) to visit 2 ($10.7 \pm 2.0\%$: $p < 0.01$) and reached the nadir at visit 9 ($10.4 \pm 1.5\%$; $p < 0.001$) (table III). Thereafter, values rose and were not significantly different from baseline at visits 11 and 12. The rise at visit 12 (to $11.0 \pm 1.9\%$) was significant compared with values at visit 9 ($10.4 \pm 1.5\%$; $p < 0.005$) (fig 1).

Blood glucose

Mean blood glucose concentrations did not alter over the six months (table III). Values after lunch were significantly increased at visits 7, 9, and 11 compared with the initial assessment, whereas values after the evening meal showed a fall, achieving significance at visits 8-11 (table IV). Blood glucose concentrations did not change significantly at other times.

Serum cholesterol

Serum cholesterol concentrations fell over the six-month study period (initial value 6.3 ± 1.4 mmol/l (244 ± 54 mg/100 ml), final value 5.7 ± 1.3 mmol/l (221 ± 50 mg/100 ml); $p < 0.001$).

Plasma urea and creatinine, and urine creatinine

There was no significant change in either plasma urea (initial value 5.1 ± 1.1 mmol/l (31 ± 7 mg/100 ml), final value 5.4 ± 1.2 mmol/l (33 ± 7 mg/100 ml)) or plasma creatinine concentrations (initial 74 ± 12 μmol/l (0.84 ± 0.14 mg/100 ml), final 76 ± 13 μmol/l (0.86 ± 0.15 mg/100 ml)) over the six months. Mean 24-hour urinary creatinine excretion was also unchanged (initial 11.6 ± 3.3 mmol/24 h (1.3 ± 0.4 g/24 h), final 12.1 ± 3.0 mmol/24 h (1.4 ± 0.3 g/24 h)).

Duration of diabetes and plasma C-peptide values

The overall duration of diabetes in the group was 15 ± 8 years (range 1-32). Only six patients had measurable circulating C-peptide concentrations. In these patients the mean duration of diabetes was less than in the remainder of the group (6 ± 4 *v* 16 ± 7 years; $p < 0.001$). While measures of control were usually nearer normal in patients with positive C-peptide results, the small number in this group precluded meaningful statistical analysis. Trends were similar to those in patients with negative results. In particular, review of the data for glycosylated haemoglobin after removal of patients with positive C-peptide results showed no change from the pattern previously described for the whole group.

Frequency of symptomatic hypoglycaemia

There was no significant change in the number of hypoglycaemic episodes during the study (table V).

TABLE V—*Frequency of hypoglycaemia during optimisation period*

No of hypoglycaemic episodes reported over preceding two weeks	No of patients			
	Visit 1	Visit 5	Visit 9	Visit 12
0	24	24	26	17
1	10	14	9	9
2	7	3	3	7
3	0	1	2	0
4	1	0	2	0
5-10	1	1	1	1
>10	0	0	0	0
No of patients with record available	43	43	43	34

Body weight, insulin dose, and carbohydrate intake

For the group as a whole there was no significant change in body weight (initial weight 66.2 ± 10.7 kg, final weight 66.4 ± 9.9 kg) or mean total daily insulin dose (initial dose 53 ± 20 units, final dose 55 ± 17 units) over the study period. Nevertheless, significant changes did occur in the distribution of insulin dose through the day. Thus when initial and final visits were compared the mean dose of short-acting insulin fell in the morning (18 ± 9 units *v* 13 ± 6 units; $p < 0.001$), while the mean dose of intermediate-acting insulin rose both in the morning (9 ± 8 units *v* 15 ± 9 units; $p < 0.001$) and in the evening (14 ± 8 units *v* 17 ± 7 units; $p < 0.005$).

The mean estimated total dietary intake of carbohydrate fell slightly but significantly from 176 ± 45 g/day at the initial visit to 166 ± 48 g/day at the final visit ($p < 0.001$).

CROSS-OVER PERIOD (NINE MONTHS)

Comparisons between different methods of self-monitoring

Table VI gives the values for glycosylated haemoglobin, 24-hour urinary glucose excretion, median Diastix scores, and mean blood glucose estimations. Results were those at the end of the six-month "run-in" period of optimisation of control with urine tests and during the cross-over period after self-monitoring by semiquantitative urine tests, visual assessment of glucose strips, and assessment with reflectance meter, irrespective of their order during the cross-over study. There was no significant change from initial values (end of run-in period) in any of these estimates of control, and no differences were found among the individual methods of self-monitoring. Examination of values at individual clinic visits (fig 2) also showed no change in glycosylated haemoglobin or in urine glucose excretion during self-monitoring by any method over the cross-over period.

TABLE IV—*Blood glucose concentrations (mean ± SD) at each individual time-point during optimisation period. Values given in mmol/l*

Visit No	Fasting		Before lunch		Before evening meal		Before bed		After breakfast		After lunch		After evening meal	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
2	23	10·0 ± 5·5	27	6·7 ± 5·4	34	9·5 ± 4·0	26	6·2 ± 3·4	14	14·1 ± 5·2	14	7·8 ± 2·1	11	13·5 ± 3·3
3	31	10·1 ± 4·7	36	6·4 ± 4·0	41	10·6 ± 4·9	36	7·3 ± 4·2	11	12·8 ± 4·6	18	9·5 ± 3·6	20	11·4 ± 6·0
4	34	10·1 ± 4·1	32	6·7 ± 3·7	34	9·9 ± 5·0	37	7·4 ± 4·5	22	11·1 ± 5·0	19	9·1 ± 3·0	15	9·9 ± 2·8
5	38	11·3 ± 6·0	39	7·0 ± 5·2	38	9·9 ± 4·6	37	7·3 ± 4·0	25	13·2 ± 5·5	28	8·4 ± 4·0	24	10·3 ± 4·3
6	41	11·2 ± 5·6	39	6·1 ± 4·5	43	10·1 ± 5·1	35	6·9 ± 4·2	27	13·0 ± 5·8	25	8·4 ± 3·4	26	10·4 ± 5·2
7	43	10·4 ± 4·5	41	6·3 ± 4·8	41	9·4 ± 4·8	42	7·0 ± 3·7	28	12·7 ± 5·5	31	9·3 ± 4·2*	24	9·4 ± 5·0
8	39	10·8 ± 5·3	44	6·9 ± 4·9	42	9·9 ± 4·5	42	7·6 ± 4·5	32	12·8 ± 5·7	33	9·8 ± 4·6	27	8·9 ± 4·6**
9	39	8·4 ± 4·0	40	6·9 ± 4·3	39	8·2 ± 4·6	41	7·2 ± 4·5	28	12·9 ± 4·5	25	9·6 ± 4·9*	24	8·7 ± 4·9***
10	41	10·3 ± 4·3	38	7·0 ± 5·2	38	10·3 ± 5·5	39	8·2 ± 5·3	30	12·4 ± 5·7	26	10·8 ± 4·8	28	11·4 ± 5·8*
11	41	9·2 ± 3·8	40	7·2 ± 5·5	38	9·0 ± 4·8	37	7·4 ± 4·8	29	11·3 ± 5·1	26	10·1 ± 3·9*	26	8·7 ± 5·7**
12	28	9·8 ± 5·5	29	7·3 ± 5·9	28	9·5 ± 5·8	28	7·9 ± 5·1	19	14·0 ± 6·7	20	10·0 ± 5·9	19	8·8 ± 5·9

Comparison with values at visit 2: *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ (Student's paired *t* test).
Conversion: SI to traditional units—Blood glucose: 1 mmol/l ≈ 18 mg/100 ml.

TABLE VI—*Results at induction to cross-over study and after 12 weeks of monitoring of control with urine tests, blood glucose values read visually, and blood glucose values read by meter. Values expressed as means ±SD or as medians (and range)*

	Induction to cross-over study	After 12 weeks of monitoring		
		Urine tests	Visual blood glucose	Meter blood glucose
Glycosylated haemoglobin (%)	10·8 ± 1·8	10·5 ± 2·0	10·6 ± 2·1	10·4 ± 1·9
Urinary glucose (mmol/24 h)	81·5 (0-685)	59 (1-903)	82 (0-680)	84 (0-1009)
Diastix score	1·5 (1-5)	1·4 (1-6)	1·4 (1-6)	1·5 (1-6)
Mean preprandial blood glucose (mmol/l)	7·9 ± 2·9 (n = 35)	8·6 ± 3·6 (n = 38)	8·0 ± 2·9 (n = 32)	8·0 ± 3·6 (n = 33)
Mean preprandial and postprandial blood glucose (mmol/l)	8·3 ± 2·4 (n = 24)	9·2 ± 3·3 (n = 25)	8·2 ± 2·6 (n = 23)	8·4 ± 3·0 (n = 21)
Serum total cholesterol (mmol/l)	5·6 ± 1·2	5·5 ± 1·1	5·6 ± 1·1	5·5 ± 1·1
Plasma urea (mmol/l)	5·5 ± 1·2	5·3 ± 1·2	5·5 ± 1·1	5·4 ± 1·2
Plasma creatinine (μmol/l)	76 ± 13	82 ± 13**	82 ± 16**	82 ± 13**
Frequency of hypoglycaemia per fortnight	0·5 (0-3)	0·4 (0-7)	0·4 (0-10)	0·7 (0-4)
Total insulin dose (units/day)	54 ± 15	54 ± 16	53 ± 18	54 ± 17
Body weight (kg)	66·7 ± 9·4	67·2 ± 10·7	67·7 ± 10·2	67·2 ± 10·8
Dietary carbohydrate (g/day)	173 ± 49	171 ± 50*	169 ± 52*	170 ± 50

Comparison with values at induction: *p < 0·05; **p < 0·01. All other comparisons not significant.
Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h. Blood glucose: 1 mmol/l ≈ 18 mg/100 ml. Cholesterol: 1 mmol/l ≈ 38·7 mg/100 ml. Urea: 1 mmol/l ≈ 6·02 mg/100 ml. Creatinine: 1 μmol/l ≈ 0·0113 mg/100 ml.

The design of the study produced six subgroups depending on the sequence of using the three monitoring methods (table I). Figure 3 shows the mean glycosylated haemoglobin values for each of these at each clinic visit. No monitoring method was associated with any particular trend in values. Past use of a meter did not improve results when using visual interpretation of strips.

Serum cholesterol concentration was not influenced by any method of self-monitoring (table VI). Plasma urea concentrations were also unchanged but plasma creatinine values rose slightly but significantly during the cross-over study, independently of the means of control ($p < 0·01$) (table VI).

The frequency of symptomatic hypoglycaemia was low and not influenced by any method of self-monitoring (table VI).

There were no significant differences in total daily insulin dose, body weight, or dietary carbohydrate intake among the three different monitoring techniques. Dietary carbohydrate intake at induction to the cross-over study was 173 ± 49 g/day and showed a slight fall with each monitoring method (urine testing 171 ± 50 g/day ($p < 0·05$), visual testing 169 ± 52 g/day ($p < 0·05$), meter testing 170 ± 50 g/day (NS)).

Of the 38 patients, 37 completed the questionnaire. No patient thought that urine testing was ideal for monitoring control (table VII), in contrast to 21 patients (57%) who believed that blood testing alone was superior. Fifteen patients (40%) thought that blood and urine testing together was better. All but one of the patients (97%), therefore, thought blood testing, either alone or in combination with urine tests, was *ideal* for controlling their diabetes. Eight patients (22%), however, thought that urine tests were the most practical means of self-monitoring, blood tests alone were considered to be the most practical by 14 (38%), and a combination of both methods was preferred by 15 patients (40%). Thus 29 patients (78%) thought that blood testing was a useful, *practical* technique.

No clear preference was expressed for blood glucose monitoring by visual inspection of the strip (20 patients, 54%) as opposed to meter reading (16 patients, 43%). One patient was ambivalent.

Three months after the study, 31 patients (84%) were still regularly carrying out home blood glucose testing, though less frequently than during the study period, only 16 patients (43%) testing more than five samples a week.

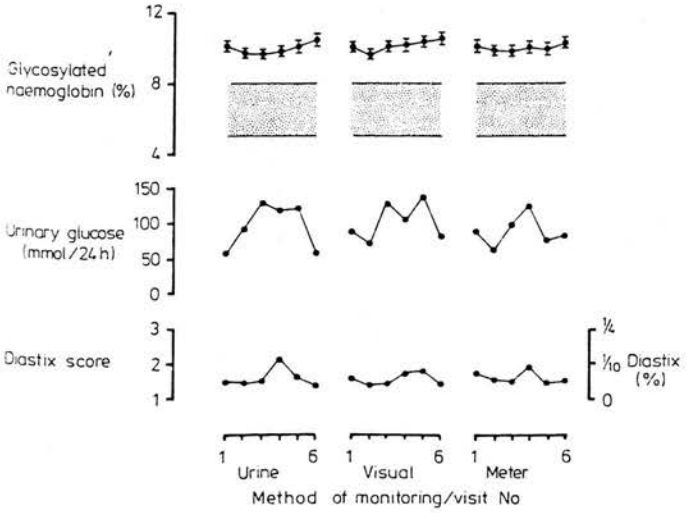

FIG 2—Results for glycosylated haemoglobin (mean ± SEM), 24-hour urinary glucose excretion (median), and median Diastix score (median) at each visit in cross-over period, irrespective of sequence of using three methods of monitoring. Shaded area indicates normal range for glycosylated haemoglobin (5·0-8·2%). Visits made two-weekly.
Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h.

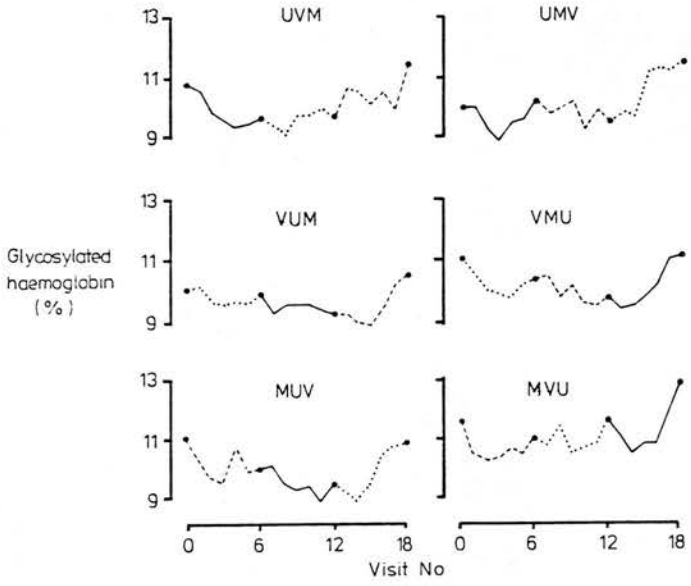

FIG 3—Results for mean glycosylated haemoglobin at every visit in each of six subgroups of patients showing method of monitoring control. Urine testing (U ●——●); visual monitoring of blood glucose (V ● . . . . . ●); meter monitoring of blood glucose (M ●— — — — ●). Visits made two-weekly.

TABLE VII—*Patients' opinions of "best" and "most practical" methods of monitoring control of their diabetes. Results expressed as number (%) of patients (n = 37)*

	"Best"	"Most practical"
Urine tests	0	8 (22)
Blood tests	21 (57)	14 (38)
Combination of urine and blood	15 (40)	15 (40)
None	1 (3)	0

Chronological analysis

Data from the study were also analysed chronologically independently of the method of monitoring. Table VIII gives the results for the end of each cross-over period—that is, three, six, and nine months.

Glycosylated haemoglobin values fell from 10·8±1·8% at induction to the study to 10·2±1·4% at three months ($p<0·02$) and to 9·9±1·7% at six months ($p<0·005$ *v* induction values). By the end of the study, at nine months, values had, however, risen to 11·4±2·4% ($p<0·001$ *v* six-month value) (table VIII).

No significant changes occurred in 24-hour urinary glucose excretion, median Diastix score, or mean blood glucose or serum cholesterol concentrations (table VIII). Although the rise in glycosylated haemoglobin values at nine months was reflected in trends to increased 24-hour urinary glucose excretion and median Diastix score, these changes were not significant.

Daily dietary carbohydrate intakes were less than initial levels (173±49 g) at six months (169±50 g; $p<0·05$) and nine months (169±51 g; $p<0·05$). There were no significant differences in total daily insulin dose or body weight at any time point (table VIII).

Figure 4 shows the changes in mean glycosylated haemoglobin values, median 24-hour urinary glucose excretion, and median Diastix score for each of the individual visits analysed chronologically. No trends were evident between cross-over points.

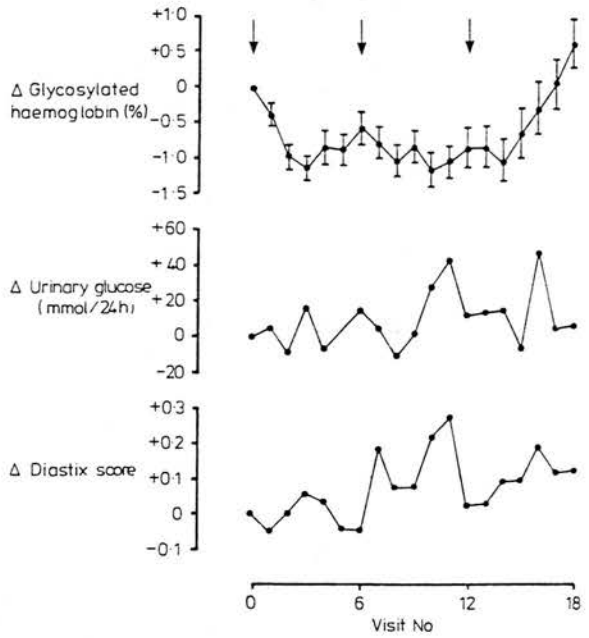

FIG 4—Results for mean change (±SEM) in glycosylated haemoglobin, median change in 24-hour urinary glucose excretion, and median change in median Diastix score over nine-month cross-over period (18 visits). Results shown chronologically irrespective of method of monitoring. Monitoring methods changed at visits 0, 6, and 12 (arrowed). Visits made two-weekly.

Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h.

Discussion

Assessing the quality of diabetic control in insulin-dependent outpatients is difficult,[29] and we therefore used multiple objective measurements. The inaccuracies associated with semiquantitative urine glucose analyses performed by the patient[9 29] may be offset considerably by measurement of multiple samples to provide an index of control. Twenty-four-hour urinary glucose excretion may be accurately measured and correlates well with mean blood glucose concentration assessed by continuous analysis,[10] but this provided only an intermittent index in this study. The unchanged urinary creatinine excretion confirmed that collections were consistent. Even frequent blood glucose samples may be relatively unreliable indices of overall control in insulin-dependent diabetes.[29-31] Estimation of glycosylated haemoglobin values provides a reliable measure of mean blood glucose concentration over the weeks before sampling, despite some difficulties with the method and interpretation of results.[32-36] Finally, since poor control may be associated with hyperlipidaemia,[37] measurement of blood lipid values may give further information on control.

Frequent outpatient visits and intensive education together with conventional urine glucose analysis were associated with significant improvements in quantitative and semiquantitative urinary glucose, glycosylated haemoglobin, and total serum cholesterol values. Other workers have failed to show objective improvements after such efforts.[7 8] This may have been due to the inadequacy of their methods of assessing control, since in our study frequent but intermittent blood glucose estimations also failed to reflect the change in control. Compliance in producing capillary blood for laboratory glucose analysis was never good, especially at the beginning of the study and after meals (table IV), so that some improvement may have gone unobserved. The change in serum cholesterol values was probably attributable to better diabetic control, since we did not attempt to change the nature or content of dietary fat.[38-40]

In the first six months of the study symptomatic hypoglycaemia before lunch and pronounced fasting glycosuria were commonly recorded, resulting in redistribution of the insulin dose with more emphasis on intermediate-acting insulin. Subsequently laboratory blood glucose values confirmed that these changes were appropriate (table IV), but the effect on blood glucose concentrations was disappointing. The deterioration noted after lunch and the improvement after the evening meal may have resulted from the change in insulin distribution. Others have noted the difficulty in controlling postprandial hyperglycaemia, even in diabetics in hospital.[41] Our results show the need for better methods of controlling fasting hyperglycaemia.

It has been strongly suggested from the earliest studies of home blood glucose monitoring[14-19] that use of this technique results in a dramatic improvement in glycaemic control. Only one study has failed to support this.[42] In our group of patients self-monitoring of blood glucose concentrations, visually or by meter, produced no further improvement in control than that achieved by regular urine tests and intensive education.

TABLE VIII—*Results at induction to cross-over study and after three, six, and nine months, irrespective of monitoring method used. Values expressed as means ±SD or as medians (and range)*

	Induction to cross-over study	Three months	Six months	Nine months
Glycosylated haemoglobin (%)	10·8±1·8	10·2±1·4**†	9·9±1·7††***	11·4±2·4
24-Hour urinary glucose (mmol/24 h)	81·5 (0-685)	72·5 (0-1009)	76·5 (0-903)	105 (1-809)
Diastix score	1·5 (1-5)	1·3 (1-6)	1·4 (1-6)	1·8 (1-6)
Mean preprandial blood glucose (mmol/l)	7·9±2·9 (n=35)	8·8±3·2 (n=34)	7·3±2·9 (n=35)	8·5±3·8 (n=34)
Mean preprandial and postprandial blood glucose (mmol/l)	8·3±2·4 (n=24)	8·8±2·7 (n=24)	8·0±2·8 (n=25)	9·2±3·5 (n=20)
Serum total cholesterol (mmol/l)	5·6±1·2	5·6±1·2	5·6±1·1	5·4±1·0
Total insulin dose (units/day)	54±15	53±15	55±17	53±19
Body weight (kg)	66·7±9·4	67·0±10·1	67·2±10·9	67·8±10·8
Dietary carbohydrate (g/day)	173±49	172±51	169±50*	169±51*

Comparison with values at induction: *$p<0·05$; **$p<0·02$; ***$p<0·005$.
Comparison with values at nine months: †$p<0·002$; ‡$p<0·001$. All other comparisons not significant.
Conversion: SI to traditional units—Urinary glucose: 1 mmol/24 h ≈ 0·18 g/24 h. Blood glucose: 1 mmol/l ≈ 18 mg/100 ml. Cholesterol: 1 mmol/l ≈ 38·7 mg/100 ml.

There are several possible reasons for the discrepancies between our own and earlier results. Our subjects were recruited by letter without prior knowledge of the patient or his quality of control, and medical reasons for exclusion (pregnancy, renal failure, etc) were clearly defined at the outset. Before starting home blood glucose monitoring all of our patients had their control optimised so far as possible by conventional means with intensive education and regular home urine testing; this is particularly important in view of the improvements noted in our run-in period. In the cross-over period each patient served as his own control. In contrast, other investigators have selected their patients for specified (or unspecified) reasons but, perhaps more important, not made intensive efforts to optimise control by conventional means and not included control groups.[43]

There may be other reasons for the disparity between this and other reports. Though our patients were well motivated, they did not monitor blood glucose values *every* day as in certain previous studies, and this intensity of monitoring may be necessary for full benefits to accrue.[44 45] Some workers[14 44] have also used multiple (more than two) injections of insulin daily, whereas our patients were receiving twice-daily injections. Also all but six of our patients gave negative C-peptide results and thus may have been less responsive to the benefits of home blood glucose monitoring. The small number of patients with positive C-peptide results did not permit meaningful comparisons but we could see no obvious differences in trend between those with positive and negative results. Work by Seigler *et al*[46] suggests that in the short term (two months) excellent control may be achieved in patients with negative C-peptide results taking twice-daily insulin provided intensive monitoring and education is used.

Schiffrin and Belmonte, who used four injections daily *and* obtained seven capillary blood glucose samples *every* day, achieved excellent glycaemic control over six months in a group of 16 patients with negative C-peptide results.[47] Unfortunately they did not define their selection procedure. They also showed that reducing the frequency of capillary blood glucose sampling to twice daily resulted in a deterioration in control. We specifically tried to place only realistic demands on our patients, since we have not been able to persuade patients to adopt the intensity of management used by Schiffrin and Belmonte.

Though home blood glucose monitoring did not appear to improve control in our patients, certain selected groups may find it helpful. Real benefit may, for example, occur in pregnancy (though this is contrary to the findings of one study[42]) in the presence of altered renal threshold for glucose or unstable control with recurrent hypoglycaemia.

Our patients greatly preferred home blood glucose measurements to urine tests alone. The importance of this should not be underestimated. Most of our patients, as in other studies,[14–19 44–47] derived a considerable amount of confidence and knowledge from the ability to measure their own blood glucose concentrations whenever required. This was particularly true with testing before bedtime. In the long term the preference for blood as opposed to urine tests might lead to a greater awareness and understanding of the importance of control by the patient, though this requires confirmation in long-term studies. The use of a meter, however, appears unnecessary, since it produced no better control and was not preferred by our patients when compared with visual readings.

In both the optimisation and cross-over periods there was evidence for an initial improvement in control with a subsequent deterioration. We cannot exclude the possibility of a seasonal variation[48] in the control of our diabetics but think it more likely to have been due to an initial enthusiasm under study conditions which subsequently waned. The crossover nature of the home blood glucose monitoring period prevented this from influencing our results and is lacking in other studies. Our experience shows the difficulty in maintaining enthusiasm over prolonged periods, which has been noted by others.[3] The short-term improvement but long-term deterioration in control shown in our chronological analysis emphasises the need for studies to be adequately controlled and of sufficient duration to allow for this problem.

Opponents of the policy of striving for improved control of blood glucose concentrations have pointed out the possibility of increased morbidity associated with hypoglycaemia.[13] The improved control with education and regular urine tests was not obtained at the price of more frequent hypoglycaemia, though episodes may have gone unrecognised. In the cross-over period urine and blood glucose monitoring resulted in similar incidences of hypoglycaemia.

The highly subjective nature of dietary assessment makes interpretation of such data speculative. The surprising levels of statistical significance for dietary changes, particularly in the cross-over period, were most unlikely to be of clinical importance and simply reflect the powerful nature of paired testing.

We conclude that in unselected insulin-dependent subjects intensive education associated with regular urine glucose monitoring can improve diabetic control. The addition of home blood glucose monitoring, though popular with patients, does not necessarily result in improved control. Results may be different with more intensive monitoring methods or in different groups of patients. The main benefit of home blood glucose monitoring probably lies in its use as an educational modality, the increased contact time with staff that automatically ensues, and the improved motivation that results from introducing any new tech nique. Enthusiasm for any method of self-assessment, however, diminishes with time. Future studies should concentrate on long-term results and must incorporate appropriate control groups.

We are grateful to the Scientific and Research Committee of Newcastle Area Health Authority (Teaching), the British Diabetic Association, and Boehringer Corporation (London) Ltd for financial support. PDH was supported by Novo Industri A/S. We acknowledge with thanks the laboratory help of Dr A Skillen, Dr P Smith, and Ms J Harrison. Ms S Beattie provided invaluable dietary advice. We gratefully acknowledge the expert work of Ms M Cooper in computating data. Owen-Mumford Ltd and Sherwood Medical Products kindly donated supplies of Autolets and Monolets. We thank Ms J Stothard, Ms B Snowdon, and Ms E Murat for skilled secretarial help.

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(*Accepted 6 August 1982*)

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Fluoride and Bone Disease in Patients on Regular Haemodialysis

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Patients treated by RDT at Newcastle commonly develop a disabling form of bone disease. The prominent clinical features are bone pain, myopathic weakness and liability to pathological fracture (Schorr, 1968; Floyd et al, 1969; Siddiqui & Kerr, 1971). Radiographs show juxta-articular bone thinning, altered bone structure, cortical thinning and Looser's zones or complete fractures (Simpson et al, 1971). Bone biopsies display diminishing osteitis fibrosa, persistent osteomalacia and progressive osteoporosis (Ellis & Peart, 1971).

The Dublin meeting of this Society drew attention to the contrast between this pattern of disease and the much lower incidence of mild bone disease at Montreal (Kaye, 1968, 1969), and Fulham Hospital, London (Curtis et al, 1968, 1969). A similar contrast between Iowa City (high incidence of bone disease) and Rochester, Minnesota (low incidence) was revealed by the meeting of the American and British Colleges of Physicians in April, 1968. In 1969 Posen described the high prevalence of osteodystrophy at Ottawa and suggested that it might be due to the use of fluoridated dialysis fluid (Medical Tribune, 1969), a possibility first proposed by Taves, Terry, Smith and Gardner (1965). We followed up this suggestion as Newcastle and Iowa City also fluoridated their tap water while Montreal, Rochester and London did not. Last year we reported that the average patient in Newcastle took up 30 mg of fluoride per dialysis and had a rising serum fluoride which correlated closely with the severity of his bone disease (Siddiqui et al, 1970). In the discussion of this paper, however, Robinson pointed out that East Birmingham Hospital had used fluoridated dialysis fluid since 1964 and had little trouble with bone disease.

We therefore undertook a comparison of patients at Newcastle and Birmingham to test the validity of our clinical impressions and to see whether any differences which emerged could be explained by variations in the fluoride content of dialysis fluid or in fluoridaemia.

METHODS

Ninety-four patients on RDT from Newcastle were compared with 42 patients on RDT from East Birmingham. Samples of tap water (softened if a softener was in use) and dialysis fluid were obtained from each of the homes and hospital centres in both cities. Pre-dialysis blood samples were obtained simultaneously for estimation of serum fluoride. All fluoride estimations were carried out by the method of Fry and Taves (1970) using the Orion Model 401 fluoride meter as previously described (Siddiqui et al, 1970).

The presence or absence of bone pain was assessed by means of a standard questionnaire distributed to all patients.

Skeletal surveys are carried out at 6 monthly intervals on Newcastle patients; the survey nearest in time to the fluoride estimation was chosen for analysis. Skeletal surveys were performed close to the time of fluoride estimation in Birmingham patients. All radiographs were studied by one radiologist and graded according to three sets of criteria (Table I). The 'juxta-articular score' is an arbitrary grading of the degree of rarefaction

Table I. Criteria for grading radiological changes

"Osteoporosis"	
0	– 1. Juxta-articular score less than 2/4. 2. Medullary score of 0/4
I	– 1. Combined score of 2/8 or 3/8 – or: 2. Medullary score of 1/4.
II	– 1. Combined score of 4/8 or 5/8 – or; 2. Medullary score of 2/4.
III	– 1. Combined score of greater than 5/8 or: 2. Medullary score greater than 2/4.
"Osteomalacia"	
0	– No fractures or Looser zones.
I	– 1 or 2 rib fractures, no fractures or Looser zones elsewhere
II	– 1. Multiple rib fractures but less than 12 in total or: 2. Other minor fractures or Looser zones.
III	– 1. 12 or more rib fractures or: 2. Femoral neck or other major fractures with deformity.
"Osteitis Fibrosa"	
I	– Equivocal erosions or sclerosis in the spine without erosions.
II	– Unequivocal erosions only.
III	– Erosions with typical changes elsewhere in the skeleton.

around the joints of the hand. The 'medullary score' is derived from the cortical thickness in the third metacarpal bone.

The radiological findings have been grouped under the headings 'osteitis fibrosa', 'osteoporosis' and 'osteomalacia' since we believe that these pathological processes are most closely mirrored by the radiological signs that we have quoted. However, this belief has not been fully validated by comparison with simultaneous histology, so the terms have been kept in inverted commas and are used as convenient contractions. For each of these characteristics a 'mean severity index' was calculated by dividing the sum of the grades by the number of patients.

RESULTS

Total groups

Bone symptoms were much commoner in Newcastle than in Birmingham patients although the latter had been dialysed for much longer on average. It was also our strong clinical impression that Newcastle complaints were more severe.

Table II. Newcastle vs Birmingham patients (Total group)

		Newcastle	Birmingham	
No. of patients		94	42	
Incidence of Bone Symptoms		36%	12%	$p < 0.005$
Mean Duration of Dialysis (weeks)		40 (1 - 348)	81 (5 - 239)	$p < 0.001$
Incidence of X-ray abnormalities		89%	90%	Not significant
'Osteoporosis':	Incidence	88%	90%	Not significant
	Severity	1.49	1.28	Not significant
'Osteitis fibrosa':	Incidence	21%	29%	Not significant
	Severity	0.26	0.48	$p < 0.02$
'Osteomalacia':	Incidence	22%	2%	$p < 0.01$
	Severity	0.47	0.05	$p < 0.01$
Serum Fluoride µg/ml		0.22 ± 0.12	0.19 ± 0.13	Not significant
Dialysate Fluoride	0.5 ppm	73*	21*	
	0.5 ppm	21	16	

*High Dialysate Fluoride Group

The total incidence of radiological abnormalities was high in both groups, but followed different patterns. 'Osteoporosis' was of similar degree in both centres but this is a phenomenon which increases with the time on dialysis, at least in Newcastle. Since Birmingham patients had been dialysed longer we must assume that their bones thinned more slowly.

'Osteitis fibrosa' on the other hand was commoner and more severe in Birmingham. However, the most dramatic difference was in the coincidence of fractures which were common in Newcastle while only one patient in Birmingham had a single Looser's zone.

The dialysis fluid fluoride estimations showed that about a quarter of Newcastle patients and nearly half the Birmingham patients had moved to home dialysis in areas with low tap water fluoride. We therefore repeated the analysis, confining our attention to the 73 Newcastle and 21 Birmingham patients who had been treated throughout their dialysis experience with high fluoride dialysis fluid (defined as having a fluoride content above 0.5 μg/ml). The mean dialysis fluid fluoride concentration in these subgroups were: Newcastle 1.0 μg/ml (SD=0.0), Birmingham 0.8 μg/ml (SD=0.18) but the means were not significantly different.

Table III. Newcastle vs Birmingham patients
High dialysate fluoride groups

	Newcastle	Birmingham	
No. of patients	34 (36%)*	5 (12%)	Significant $p < 0.01$
Dialysate Fluoride	High 28 Low 6	3 2	–
Duration of Dialysis (wks)	118	188	Significant $p < 0.002$
Serum Fluoride μg/ml	0.28 ± 0.17	0.19 ± 0.11	Not significant
FEATURES Radiological Osteoporosis	Incidence 100% Severity 2.15	Incidence 100% Severity 2.15	Not significant
Osteomalacia	Incidence 50% Severity 1.22	Incidence 0 Severity 0	Significant $p < 0.01$
Osteitis Fibrosa	Incidence 29% Severity 0.44	Incidence 80% Severity 1.8	Not significant Significant $p < 0.01$

* (%) of the Total Group q.v. see Table I

*throughout this paper fluoride content has been expressed in micromols per ml since this is the notation used by Water Boards, 1 microgram = 53 micromols per litre. The method employed in this study measures only ionised fluoride.

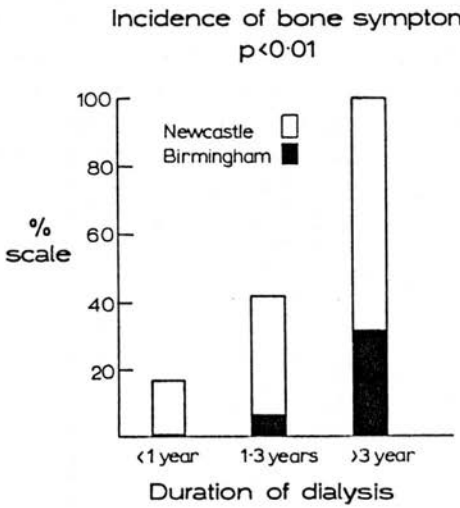

Figure 1. Prevalence of bone symptoms in Newcastle and Birmingham patients exposed to high fluoride dialysis fluid throughout

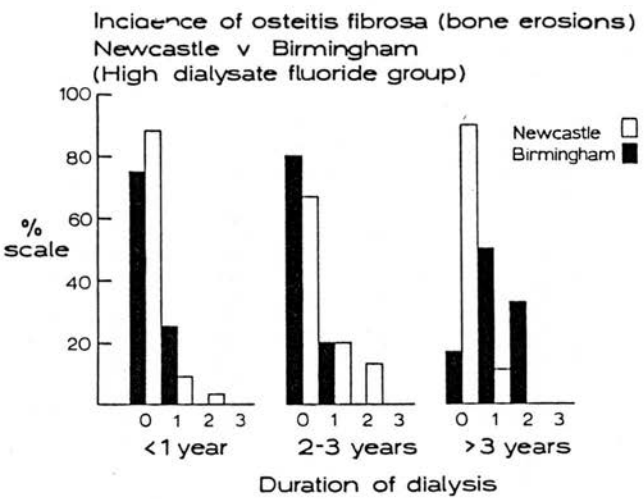

Figure 2. Prevalence of 'osteitis fibrosa' in Newcastle and Birmingham patients exposed to high fluoride dialysis fluid in this and the next two figures the numbers 0,1,2,3, on the horizontal axis refer to the scale of severity as set out in Table I. The histograms represent the percentage of patients in each grade

High Fluoride groups (Table III)

The same differences between Newcastle and Birmingham patients were found as in the total groups. Bone symptoms increased with duration of dialysis in both cities (Figure 1) but much more rapidly so in Newcastle.

'Osteitis fibrosa' was usually absent or equivocal in both centres up to the third year of dialysis (Figure 2) but thereafter more Birmingham patients developed definite signs of osteitis and the two groups were significantly different.

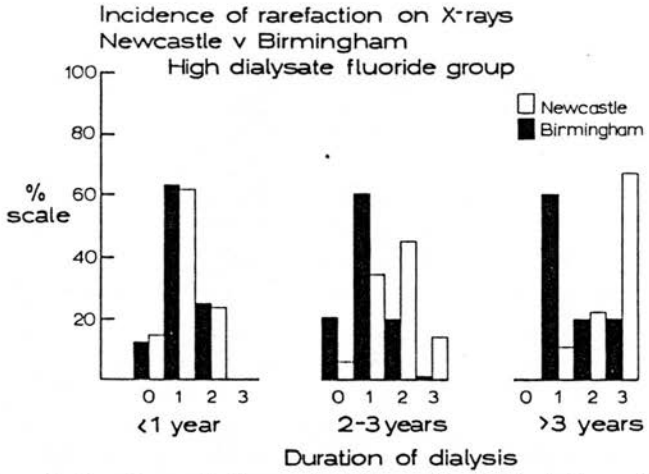

Figure 3. Prevalence of 'osteoporosis' in Newcastle and Birmingham patients exposed to high fluoride dialysis fluid

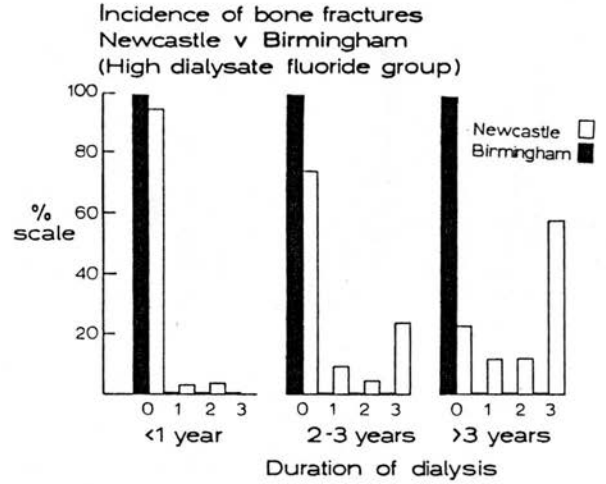

Figure 4. Prevalence of 'osteomalacia' (bone fractures) in Newcastle and Birmingham patients exposed to high fluoride dialysis fluid

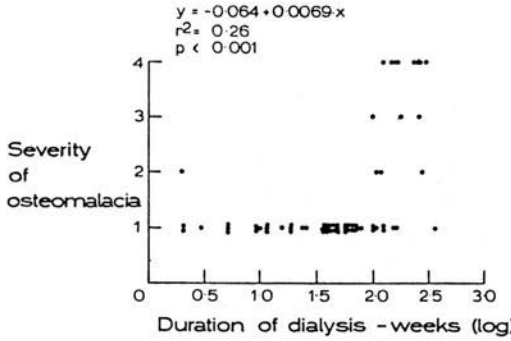

Figure 5. Relationship between severity of 'osteomalacia' and the logarithm of duration of dialysis in Newcastle patients exposed to high fluoride dialysis fluid

'Osteoporosis' was significantly related to time on dialysis in Newcastle ($r^2 = 0.31$; $p < 0.02$). There was a non-significant trend in the same direction in Birmingham. The difference between the two centres in the later years of dialysis (Figure 3) is not quite significant at the 5% level.

'Osteomalacia' is clearly different in the two cities. None of the Birmingham patients in the High Fluoride group had any fractures or Looser's zones while half the Newcastle patients beyond the third year had reached grade 3 — more than 12 rib fractures or other major fractures (Figure 4). The relationship between 'osteomalacia' and duration of dialysis at Newcastle was highly significant (Figure 5). This relationship was only slightly improved by taking serum fluoride into consideration (severity of 'osteomalacia' = 0.364 + 1.80 x serum fluoride + 0.0055 x log duration of dialysis. $r^2 = 0.30$; $p < 0.001$).

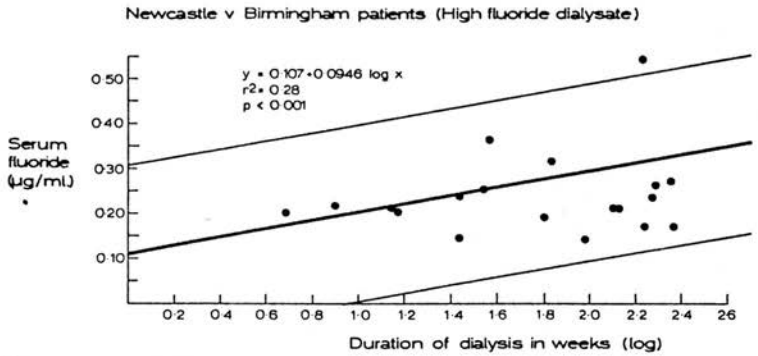

Figure 6. Relationship between serum fluoride and the logarithm of duration of dialysis in patients exposed to high fluoride dialysis fluid. The thick line is the mean regression for Newcastle patients and the thin lines are the 95% confidence limits for Newcastle patients. The individual points represent single Birmingham patients

We conclude that there is a striking difference between the patterns of bone disease in Newcastle and Birmingham even in patients exposed to the same dialysis fluoride concentration. It is however necessary to establish that these patients achieve the same serum fluoride levels in both cities. In the Newcastle group serum fluoride bears an exponential relationship to duration of dialysis (Figure 6). Of the 21 Birmingham results, 20 fell within the 95% confidence limits of the Newcastle patients and although the regression line for Birmingham was less steep than that for Newcastle the difference was not significant.

It is therefore most unlikely that any of the clinical and radiological differences between Birmingham and Newcastle patients are explained by their exposure to, or handling of, fluoride. This conclusion gains further support from a comparison of the High Fluoride and Low Fluoride groups in both centres.

Comparison of High Fluoride and Low Fluoride groups

There was no significant differences between the High and Low Fluoride groups in the incidence of bone symptoms or in any of the radiological features although serum fluoride was, as expected, significantly lower in the patients treated with low fluoride dialysis fluid. The value of this comparison is of course diminished by the varied past exposure to fluoride.

Table IV. Newcastle vs Birmingham patients
Symptomatic bone disease group

		Newcastle		Birmingham		
No. of patients		73		21		
Duration of Dialysis (weeks)		41 (1 – 348)		98 (5 – 239)		Significant $p < 0.05$
Serum Fluoride µg/ml		0.25 ± 0.11		0.24 ± 0.10		Not significant
Incidence of Bone Symptoms		46%		14%		Significant $p < 0.01$
X-ray FEATURES	Osteoporosis	Incidence Severity*	88% 1.4	Incidence Severity*	95% 1.3	Not significant
	Osteomalacia	Incidence Severity*	22% 0.51	Incidence Severity*	0 0	Highly significant
	Osteitis Fibrosa	Incidence Severity*	19% 0.25	Incidence Severity*	38% 0.48	Significant $p < 0.01$

*Mean severity index (see text)

Comparison of patients with symptomatic bone disease in the two cities (Table IV)

The differences between the centres are highlighted by looking at the worst affected patients from both cities. The typical Birmingham patient with symptomatic bone disease has been on RDT for over 3 years, has well developed 'osteitis fibrosa' with bone thinning but with no fractures or Looser's zones. The typical Newcastle patient develops symptoms earlier and often has pathological fractures in addition to bone thinning; osteitis fibrosa is not a prominent feature in Newcastle – a conclusion we have already drawn on biochemical and histological grounds (O'Riordan et al, 1970; Ellis & Peart, 1971). In both centres the characteristic pattern for that centre can develop, either in high or low fluoride areas.

DISCUSSION

The hypothesis that fluoridated dialysis fluid contributes to the development of early severe renal osteodystrophy is not supported by this study. There are obvious problems in a two-centre study of this type; the patients may have been more aware of the investigators concern about bone disease in Newcastle than in Birmingham and the radiographs were not read 'double-blind'. We cannot be certain that the bone disease status of the two groups of patients was identical at the beginning of the study though we think this highly probable in view of the similar methods of selection in the two centres. Bone disease eventually develops in both groups of patients and we cannot dismiss a possible role of fluoride in this process. Nonetheless there are striking differences in the patterns of osteodystrophy in patients on regular dialysis in Birmingham/Newcastle which cannot be explained by fluoridation practices in the two cities.

We have therefore looked for other differences in technique which might explain them; so far we have found none. The Kiil dialyser is now almost exclusively used in both cities; most are of Watson Marlow manufacture and all are assembled wet with 12 micron Cuprophane, cross grain. The majority in both cities are treated three times a week for $9\frac{1}{2}$-10 hours; a few in both hospital centres are on twice weekly 12-14 hour dialysis. The prescribed diet is 60 g protein per day in both cities, though this may not be the figure actually consumed (Frost & Siddiqui, 1971). Neither unit uses routine supplements of calcium, aluminium hydroxide or Vitamin D though a few patients in both places have received these from time to time. Heparin is usually given by infusion, in similar prescribed doses, both at Birmingham and at Newcastle. Dialysis fluid calcium is 6 mg/100 ml in both cities.

Earlier dialysis experience of the older patients differs more than present techniques. Newcastle changed to thrice weekly dialysis in its hospital unit later than Birmingham. However, the changeover has not apparently

altered the pattern of bone disease there (Frost & Siddiqui, 1971), and typical 'Newcastle bone disease' has appeared in patients treated from the start on thrice weekly dialysis in the Newcastle area.

We therefore remain attracted to the idea that some local factor, probably in the water used to make dialysis fluid, is responsible for the major differences we have demonstrated. Some support for this concept has come from the experience of one severely affected patient in Newcastle. After 6 years' dialysis she had severe bone pain, multiple unhealed fractures and muscle weakness which confined her to a wheel-chair. For the last year she has been treated by dialysis against fluid made from distilled water. During this time her bone pain has disappeared, her fractures have healed and she has learnt to walk again. Full details of this study will be published elsewhere (Siddiqui et al, 1971) and a larger scale experiment of dialysis against fluid made from distilled or deionised water in Newcastle is planned.

SUMMARY

Symptomatic osteodystrophy is uncommon in patients on RDT in Birmingham, it appears late and is associated with pronounced osteitis fibrosa. In Newcastle osteodystrophy is common and severe; it appears earlier and is associated with multiple fractures but not accompanied by radiological evidence of osteitis fibrosa. These differences cannot be explained by variations in exposure to fluoride but a pilot study suggests that some other solute in the tap water may be responsible.

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OPEN DISCUSSION

G POSEN (Ottawa): I'd like to make a few comments and ask a couple of questions. We have come to realise, in comparing our results with other centres, that there is a difference between centres. There are centres, particularly downstate in New York, who are using fluoridated water and again had the same type of results. We did the same type of work together as you did with Birmingham except that we had bone disease. But in 1968 we deionised our water, and the results have been fantastic. Since that time, we have had no bone disease; in fact there is no symptomatic bone disease in my unit in any patient up to three years on dialysis. Patients who had severe bone disease when we were using fluoridated water and non-deionised water have healed their bone disease without any problem. I feel that fluoride, as I stated in our original paper, may still have a role to play: we should not dismiss it out of hand because it is a known toxin to many enzymes in bones, particularly to the alkaline phosphatase series. But I agree with you – by itself I don't think it causes bone disease. We also found that in the days when our fluoride was high, we still had a great deal of extraosseous calcification, both within blood vessels and without. This was related primarily to levels of phosphate, and when the phosphate was controlled, these things seemed to melt by themselves.

KERR: I am delighted to hear that you've had such good results in installing deionisers because obviously, on the basis of this study, we have just ordered them. Perhaps I should add that in Seattle where they first described the bone disease problem they did use deionised water.

HOCKEN (Hull): I am interested in your description of the noxious content of the waters of Newcastle, Professor Kerr, because Newcastle is very well known in Britain for the quality of its beer. We have a common link, as you know, in a patient who has come from you to us, and that patient arrived about two years ago osteodystrophic and myopathic. She entered a perfectly routine dialysis programme which we run and is now fully rehabilitated, runs a house, can climb on buses and goes out and does her own shopping. I have studied your work with considerable interest and like yourself, can find no

difference between your dialysis programme and ours, other than that perhaps we keep our serum phosphates a little lower than you have done. Again, it would appear to point to some elusive factor, basically in the water, and I would close by asking whether you would like to study a sample of my water?

KERR: First a comment about keeping the phosphate lower. The one biochemical difference that has always emerged between Newcastle and other centres with which we have compared ourselves (like the Royal Free and Birmingham) has been a slightly higher phosphate level; but it may well be that this just reflects a fact that we've got bone disease and therefore our patients are leaking phosphate out rather than any difference in their diet. There is certainly no difference in the prescribed diet or in the use of aluminium hydroxide. I am very pleased to hear that our mutual patient has lost her bone disease and this has also been the experience of two other patients from Newcastle, who have moved out to home dialysis in different areas with different water supplies. I wish I could tell you what is the toxic factor in Newcastle tap water. You probably know that we get 20% of our water from the River Tyne, which is a notorious open sewer, full of industrial effluent. Until fairly recently there was a factory that emptied into the Tyne above our lowest water take-off point metallic effluents including, amongst other things, cadmium. This of course has been responsible for bone disease in Japan. Unfortunately for our investigations, it closed down about three years ago, so if cadmium is the cause of our bone disease, it may disappear before we even have time to install our deionisers.

E RITZ (Heidelberg): Dr Kerr, do you have any idea about the detergent concentration of the tap water in Newcastle? I am asking this because phosphonate content in the water may be of importance for the development of bone disease.

KERR: No, we haven't any measurements, but by virtue of the fact that we do draw 20% of our water supply from a river which contains sewage from higher upstream, we presumably do have a higher detergent content than Birmingham, which takes all of its water from the Welsh mountains when the Welsh Nationalists permit it to do so.

A FOURNIER (Paris): I just want to add that the experience of the Mayo Clinic supports the view that fluoride may be toxic because we compared a group of six patients on a high fluoride with a group of six patients on a low fluoride and found a significantly larger amount of osteoid in the group with higher fluoride in the blood and in the dialysate.

Nephron
Editors: G. M. BERLYNE, Beersheva; S. GIOVANNETTI, Pisa
Publishers: S. KARGER, Basel
SEPARATUM (Printed in Switzerland)

Nephron *14:* 209–227 (1975)

David Appleton 54

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Adequate Dialysis

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Abstract. Regular haemodialysis with the Kiil dialyser for 8–10 h three times a week is the present standard of adequate dialysis. In 100 patients treated by this regime there was no positive correlation between plasma urea and creatinine before or after dialysis and any of the symptoms of which these patients still complained. There are no grounds for believing that a further increase in dialysis would relieve residual symptoms. However, any reduction in current standards of dialysis should be justified by prolonged clinical trial of large groups of patients before they are accepted as equivalent in view of the infrequency of some uraemic manifestations such as pericarditis. The implications of the middle molecular hypothesis are discussed.

Key Words
Haemodialysis
Middle Molecules
Sexual Function
Renal osteodystrophy
Neuropathy
Pruritis

All current systems of dialysis fall far short of restoring the patient to clinical and biochemical normality, and any definition of 'adequate dialysis' must be arbitrary. The most popular implied definition is 'dialysis which permits prolonged survival with full working capacity'. By this definition home haemodialysis in Europe is usually adequate; survival is better than with any other method of treatment, and over 90% of patients are fully rehabilitated [9, 38, 66]. No doubt patient selection plays a part in these results, but they do entitle us to say that the systems of home dialysis in common use in Europe in the last few years are adequate by the most popular criterion. The only recorded detail of home dialysis technique is that about 80% of the patients are dialysed three times a week [38], but it is known that British centres provided more than half the European experience up to 1971 [9], and that most of these followed the example of the pioneers [6, 29, 78] who recommended 8–10 h of Kiil dialysis, three times a week. Kiil dialysis for 24–30 h/week has achieved excellent long-term survival [14, 18, 37, 61, 71, 74] and can be accepted as the current yardstick of adequate dialysis [28].

However, patients receiving this type of dialysis do not achieve 'full rehabilitation' by stricter criteria. They seldom return to heavy manual employment or full social activity. Although a few prove their fertility [16, 88], sexual fulfilment eludes the majority [30, 47, 48]. Pericarditis still strikes occasionally at patients receiving apparently adequate dialysis [72, 81]. It is our impression that defective shunt or fistula function is a common precipitant – presumably by decreasing blood flow through the dialyser – but it can occur in patients with a pre-dialysis plasma urea consistently below 150 mg/100 ml [81]. Numerous minor symptoms, of which the patient may not complain spontaneously, are revealed by detailed questionnaires. Many of these symptoms resemble those of uraemia, and it is tempting to attribute them to the incomplete correction of azotaemia achieved by even the best dialysis systems. One early study did indeed show that patients sticking strictly to a protein-restricted diet, and therefore having lower plasma urea levels, had fewer symptoms during dialysis [80], but the groups were not matched prospectively and may have differed in other respects; moreover, the dialysis schedule was shorter than the present standard of adequacy. Most subsequent investigators have simply assumed that it is desirable to keep plasma urea and creatinine below prescribed limits. For instance DE PALMA *et al.* [65], in a full discussion of adequate dialysis, suggested a number of standards which the patient should reach in quality of life, absence of neuropathy, haematocrit, dry weight, serum albumin, etc.; their first two requirements were a blood urea below 215 mg/100 ml predialysis and a serum creatinine below 14.0 mg/100 ml. Obviously a patient on a conventional dialysis schedule who has plasma levels much higher than these is either failing to stick to any sort of diet and/or receiving less dialysis than his doctor imagines because of poor shunt function, warped boards, clotting in the dialyser, etc. He is heading for trouble from inadequate removal of water, potassium, phosphate, and other molecules, whether or not the urea and creatinine are themselves harmful. However, below some such danger limit, there is still a wide variation in the pre-dialysis (and post-dialysis) plasma concentrations observed in different patients. This is due to variations in diet (in the case of urea) or the amount of dialysis prescribed per unit weight; most doctors employ a standard schedule with little flexibility, although their patients may range in weight from 40 to 80 kg.

This unintentional experiment gives us an opportunity to find out whether any of the residual symptoms of dialysis patients are due to inadequate removal of uraemic 'toxins'. We have attempted to do this on our patients before considering whether any reduction in dialysis time would be justifiable.

Materials and Methods

Kiil dialysis for 9–10 h three times a week became the standard treatment in the home in October, 1967, and in the hospital at Newcastle upon Tyne in April, 1969. During the first half of 1973 there were 100 patients receiving regular dialysis of this type – approximately half in the home or home training unit and half in hospital centres in Newcastle or Sunderland; some of the 16 patients at Sunderland received 8 h three times a week. All had been treated for at least 3 months up to 8 years. They attend a follow-up clinic every 3 (home) or 6 (hospital) months when they fill out a detailed questionnaire and are fully examined. For the purposes of this study, an additional questionnaire was employed dealing at greater length with the symptoms listed in table I, which were chosen since they are common complaints in untreated patients with uraemia.

Plasma urea, sodium, potassium, chloride, and bicarbonate were measured fortnightly pre- and post-dialysis; serum calcium, phosphate and alkaline phosphatase were measured at least monthly before dialysis. Serum or plasma creatinine was measured pre- and post-dialysis once a month in one hospital and more frequently in the others. To estimate the level of azotaemia, we therefore averaged plasma urea for the month preceding review but plasma creatinine for the month and 3-month period prior to review. The percentage of decrease in plasma urea and creatinine during dialysis were calculated.

At the outpatient visit, height and weight were measured and percentage deviation from average and ideal weight [22] were calculated. Of the findings on physical examination, only those related to neuropathy were included in this study. The patient's activities were scored: 0 = normal working capacity in previous employment; 1 = restricted working capacity but patient mobile; 2 = largely confined to home or hospital. The average weight loss during dialysis was calculated for the month prior to review.

The correlation coefficient for each pair of the biochemical variables was calculated. Groups with or without each of the symptoms listed in table I were compared in terms of each biochemical variable, using a t test. The correlation between symptoms was studied with a χ^2 test on 2×2 tables (table II). Age, height, weight, percentage deviation from average and ideal weight, and weight loss during dialysis were correlated with each of the biochemical variables and with each of the symptoms. The computer programme was written in SPSS.

Results and Interpretation

The incidence of the symptoms is shown in table I. The biochemical results are listed in table III. The incidence of some of the symptoms in our normal population is not negligible, but we have no doubt that this is far from a normal population in its symptomatology as well as its biochemistry. Physical activity was reduced in 54% and libido in 55%. Patients made their own assessment of whether their sex life was normal; in a subsequent study we have questioned them directly about frequency of intercourse and have the impression that they understate their loss of sexual activity, though no control data are avail-

Table I. Incidence of symptoms in 100 regular haemodialysis patients

Symptoms	%
Nausea	32
Vomiting	28
Tingling	29
Numbness	52
Raynaud's phenomenon	30
Weakness	33
Cramps	67
Restlessness	45
Muscle twitching	24
Itching	39
Bone pain	39
Abnormal menstruation	32% of premenopausal women
Reduced libido	55
Reduced physical activity	54
Blackouts	4
(Evidence of neuropathy)[1]	17

[1] On physical examination.

able for the local population. 17% of patients were recorded as showing some degree of neuropathy, but this included such minor findings as loss of ankle jerks, diminution of vibration, or position sense in the toes. No patient had severe or disabling neuropathy, and in none was it the limiting factor in physical activity. This has always been true of our dialysis population; neuropathy has been virtually confined to those who developed it while on conservative therapy or peritoneal dialysis; only two patients have developed disabling neuropathy while on haemodialysis out of 340 treated in the last 10 years. Other neuromuscular symptoms were commoner and more disabling; 67% complained of cramps – usually during dialysis or when dehydrated. Restlessness of limbs was most common on lying down at night or when on daytime dialysis. Bone pain was common, affecting 39% of the patients; this is also characteristic of our dialysis centre, which has a high incidence of osteomalacic bone disease [81].

Tingling and numbness in the fingers were common but not apparently related to neuropathy. They were often precipitated by cold and sometimes

[1] Throughout the text a difference or correlation is described as significant if $p < 0.05$, very significant if $p < 0.01$, and highly significant if $p < 0.001$.

Table II. An example of correlation between symptoms: tingling in the fingers and Raynaud's phenomenon[1]

Tingling	Raynaud's phenomenon absent	present
Absent	52	12
Present	13	14

[1] Missing observations: 9 (questionnaire incomplete or answer equivocal) $\chi^2 = 10.20$ with 1 degree of freedom; $p \leq 0.01$.

Table III. Biochemical parameters in 100 patients on regular haemodialysis (Watson Marlow Kiil, 8–10 h 3 times a week; dialysate sodium 130–132 mEq/l, Ca 3.0–3.2 mEq/l, K 1.7 mEq/l)

Variable	Mean	SD	Range	Distribution
Urea pre-D, mg/100 ml	146.6	36.6	68–284	+ve skew
Urea post-D, mg/100 ml	54.4	23.2	17–138	+ve skew
Creatinine pre-D, mg/100 ml[1]	12.80	2.95	7.0–25.0	+ve skew
Creatinine Post-D, mg/100 ml[1]	6.00	2.19	2.2–13.1	+ve skew
Decrease in urea, %	62.2	12.5	25–89	+ve skew
Decrease in creatinine, %[1]	53.6	10.8	24–78	−ve skew
Sodium pre-D, mEq/l	138.4	6.1	120–149	−ve skew
Potassium pre-D, mEq/l	4.96	0.90	3.0–7.3	+ve skew
Calcium pre-D, mEq/l	4.88	0.61	3.0–7.6	normal
Phosphate pre-D, mg/100 ml	5.48	2.27	0.7–12.3	normal
Alkaline phosphatase, IU	108.3	96.5	15–640	+ve skew

[1] Results shown are for creatinine in the month before study. Those for 3 months were almost identical.

confined to the shunt or fistula limb. They had a highly significant[1] correlation with other features of Raynaud's phenomenon (pallor, cyanosis in response to cold) (table II). We regard them as an indication of vascular insufficiency.

Age

None of the biochemical or clinical features correlated with age except sexual function. The mean age of those who regarded their sexual function as normal was 38.3 years and of those who regarded it as reduced 46.2 years ($p < 0.001$).

Weight

Our patients were, on average, close to 'ideal' weight for their age and height. There was a strong correlation between weight (expressed as a percentage deviation from average or ideal weight) and activity (table IV). The most active patients are the heavier ones, and it would appear that 'ideal' weight is far from ideal for the dialysis patient; he is better off near the supposedly excessive weight of the average citizen.

As expected, weight (or height) correlated closely with pre- and post-dialysis creatinine, since the amount of dialysis was not tailored to the size of the patient. Percentage deviation from ideal weight also correlated well with pre- and post-dialysis creatinine concentration, suggesting that the heavier patients were heavier mainly because of extra muscle mass, in keeping with their better activity.

Weight loss during dialysis is adjusted to equal weight gain between dialyses and is therefore a measure of fluid intake excess to requirements. It had the expected correlation with the size of the patient, judged by height, weight or percentage deviation from average weight but not with other parameters except alkaline phosphatase (presumably a spurious correlation; a few are bound to appear when so many parameters are examined).

Activity

Apart from its correlation with weight, activity correlated directly with post-dialysis urea concentration, pre- and post-dialysis creatinine and pre-dialysis potassium. The wide scatter in pre-dialysis urea reduced the significance of its correlation with other parameters. We conclude that the more active patients have higher plasma creatinine levels because of their greater muscle mass and higher plasma urea and potassium levels because of their less inhibited dietary habits or because of their lower dialysis provision relative to body mass. There is certainly no suggestion here that activity is dependent on the amount of dialysis within the limits studied.

Correlation between Symptoms

The very significant correlation between Raynaud's phenomenon and the tingling and numbness which we sought as evidence of early neuropathy has been mentioned above. We now regard tingling as usually indicating vascular insufficiency. Nausea, not surprisingly, had a highly significant correlation with vomiting. Both symptoms were significantly related to reduced physical activity; either the patient who vomits after dialysis cannot go to work, or the patient who is unemployed has time to vomit after dialysis! Tingling also cor-

related very significantly with reduced physical activity, suggesting that those with poor vasculature (or multiple shunt problems) found it difficult to work. A significant correlation between muscle weakness and reduced activity was found as expected, but we were surprised to find no such association with bone pain; presumably the majority of patients with minor bone symptoms 'soldier on', and the disabling effects of severe bone disease, which are beyond dispute, do not show up in a statistical study of a large group, which fortunately contains few patients with very bad bone disease.

With these exceptions, there were no significant correlations between these possibly uraemic symptoms, which suggests that they do not have a common aetiology such as underdialysis.

Correlation between Biochemical Values

Several significant correlations were found between urea and creatinine which suggest that the main factor determining the control of azotaemia in our patients was the amount of dialysis per unit of body mass. Plasma urea pre- and post-dialysis correlated directly with plasma creatinine pre- and post-dialysis (over both 1 and 3 months) ($p < 0.01$ or $p < 0.001$). The percentage fall in plasma urea and plasma creatinine (over both time periods) correlated directly ($p < 0.001$); each had an inverse correlation ($p < 0.01$ or $p < 0.001$) with pre-dialysis urea or creatinine. Plasma potassium pre-dialysis correlated directly with pre-dialysis plasma urea or creatinine ($p < 0.01$).

The only other finding of interest was that serum calcium varied inversely with serum phosphate and alkaline phosphatase ($p < 0.05$).

Correlation between 'Uraemic' Symptoms and Biochemical Parameters

Nausea and vomiting are apparently not uraemic manifestations in this group of patients. They had a very significant or highly significant *inverse* correlation with the level of plasma creatinine pre- and post-dialysis and urea post-dialysis. A similar trend with pre-dialysis urea did not reach significance, possibly because of its wider scatter. It would appear that the larger, more muscular patients receiving less dialysis for their size are less likely to vomit during or soon after dialysis than their smaller, thinner, and feminine fellow patients.

Nausea and vomiting in these circumstances are presumably part of the disequilibrium syndrome which has been attributed to osmotic disequilibrium between cells and extracellular fluid due to removal of urea [43] or sodium [89]. This would fit with our observation that nausea and vomiting correlated directly with the percentage reduction in plasma urea or creatinine during

dialysis. However, these are nonspecific indications of the amount of dialysis per unit body mass, so we may merely be repeating in another form the statement that larger and more muscular patients are less likely to vomit after dialysis. The observation is therefore equally compatible with other explanations of disequilibrium including the slower correction of pH in the cells and CSF than in the blood [17, 45] or tissue hypoxia due to a rapid change in the oxygen dissociation curve [39], both of which would be accentuated by faster dialysis. However, we suspect that the symptoms often have more mundane explanations, including febrile reactions to transfusion [44], pyrogens, or reduced cerebral perfusion following excessive fluid removal [23, 44]. It is our impression that the last is the commonest explanation of nausea and vomiting (sometimes with headache) in our patients; they coincide in time with the hypotension that often follows dialysis, and they appear to affect the thinner patients disproportionately. However, it is difficult to reconcile this strong clinical impression with the apparent freedom from trouble during rapid ultrafiltration for short dialysis reported by CAMBI *et al.* [12].

There was no correlation between the incidence of numbness, weakness, cramps, restlessness, muscle twitching, itching, Raynaud's phenomenon, neuropathy, blackouts, menstrual irregularity or amenorrhoea, impaired sexual function, or bone pain with any biochemical parameter.

We conclude that the residual symptoms of our patients on Kiil dialysis for 8–10 h three times a week are not dependent on their retention of urea or creatinine. The same conclusion should apply to larger molecules, since the extent of creatinine and even of urea retention was largely determined by the number of 'square-meter-hours' of dialysis per unit body mass. However, there are two aspects of uraemia which we could not examine in this study which may be exceptions to our general conclusion.

Pigmentation was not studied because of the difficulty in interpretation in a population with hair colour ranging from nordic blond through celtic red to mediterranean black, reflecting our mixed ancestry. In a separate study we have quantitated pigmentation by skin reflectometry and histology and have found a wide range of skin colour not entirely explained by variations in melanin pigmentation [COMAISH, ASHCROFT and KERR, unpublished data]. It is our impression, not validated by any statistical evidence, that pigmentation is heavier in patients on shorter dialysis, notably those receiving only 8 h Kiil dialysis three times a week. This is compatible with the work of TSALTAS [86, 87] who showed retention in the uraemic subject of pigments related to carotene and probably having a similar molecular weight of around 500. The use of very permeable membranes has been followed by a decrease in skin

pigmentation accompanied by the passage into the dialysate of a greenish fluorescent material [32]. We suspect therefore that 'adequate dialysis' by other criteria is not adequate to control pigmentation in those subjects with a propensity to develop it.

Pericarditis could not be included in our study, since none of our patients developed it. It was a fairly common complication of our dialysis programme in its early days, occurring about once every 10 patient years of exposure, but it has now become rare (only one episode in the last 100 patient years). We suspect therefore that we operate with a wider safety margin against whatever temporary factors precipitate pericarditis. An increased incidence of pericarditis might be the first indication that a shortened dialysis schedule was inadequate and prolonged observation of a large group of patients would be necessary to permit any meaningful statement; 50 patients followed for 2 years appears to us the minimum clinical trial that can justify a new dialysis schedule. A much larger sample would be necessary before it could be stated than the incidence of pericarditis was not increased, with statistical significance.

Discussion

The interpretation of our study, and of all trials of new dialysis schedules, is bedevilled by the multiple sources of morbidity to which dialysis patients are exposed (fig. 1). Any of the 'uraemic' symptoms we studied can be produced by factors other than underdialysis. Bone pain and the associated muscle weakness and impaired activity, which are important sources of morbidity in our patients, may be related to variations in phosphate intake [82], use and abuse of aluminium hydroxide [21, 40], intake of vitamin D [7] and its metabolism in damaged kidneys [55] rather than to control of uraemia. Anaemia and its attendant fatigue (which in turn influences sexual activity) may be more dependent on external blood losses from inadequate washback, needling of fistulae, blood sampling, etc. than on azotaemia [41, 50]. Dialysis brings no immunity from ageing, and morbidity rises rapidly with advancing age [14, 34, 67]. Many of the side effects of accumulated drugs imitate those of uraemia and may differ from those encountered in subjects with normal renal function; e. g. androgens can cause muscle wasting rather than hypertrophy [49].

Assessment of subjective symptoms is also dependent on the psychological state of the patient. Some of the psychological stresses to which the dialysis patient is subjected are illustrated in figure 2, from the personal experience of EADY [25].

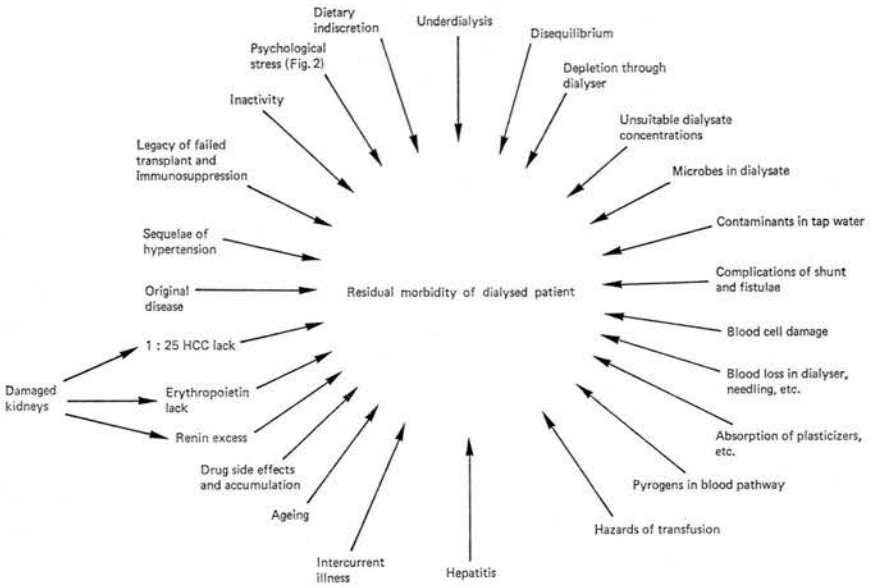

Fig. 1

Consequently most investigators studying new dialysis schedules have selected one aspect of uremia which can be assessed objectively. The most popular has been neuropathy – partly because slowing of nerve conduction may precede clinical neuropathy and can be expressed quantitatively. It is not an ideal parameter, because of the errors in nerve conduction and its uncertain relationship to clinical features [46] and because neuropathy often originated before dialysis [42, 62], may progress very rapidly [42, 84], but improves very slowly and may be caused by factors other than underdialysis including rapid dehydration [10, 57], hepatitis [20], other infections [84], and antibacterials. Encephalopathy is a more appropriate phenomenon to study since it changes over a shorter time scale, is present (as judged by EEG) during nearly adequate dialysis [44, 59], and can be measured by reproducible clinical tests [58, 85] as well as the computer-analysed EEG [44, 59]. However, it is also subject to interference by other factors such as fever [44], hypotension, disequilibrium, other metabolic disturbances, and dialysis dementia [2, 52] of possibly toxic origin [73].

Many new dialyser schedules, involving the use of large surface area or highly permeable membranes, are currently undergoing clinical trial [8, 11, 26,

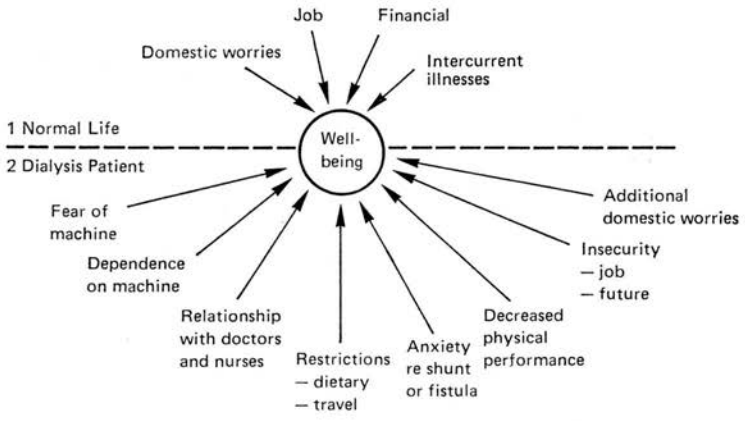

Fig. 2. Psychological causes of symptoms in the patient on regular haemodialysis. Reproduced from EADY [1971] by permission.

53, 56, 64, 79]. In view of all the difficulties we have listed we advise great caution in accepting any of these as a substitute for the well tested regime of Kiil dialysis for 8–10 h three times a week before the new schedules are established by prolonged experience in the hands of their originators.

The Middle Molecular Hypothesis

During the mid-1960s SCRIBNER [75] suggested that the relative well-being of patients on regular peritoneal dialysis, with a low-urea clearance, might be explained by the passage of higher molecular weight substances through the peritoneum. SHALDON [77] gave a similar explanation for the better survival of patients treated by regular haemodialysis with thin Cuprophan. The 'middle molecular hypothesis' that many of the features of uraemia were due to accumulation of substances in the MW range 200–1500 resulted and has survived the collapse of its twin foundations: there is no longer any difference in survival or rehabilitation between patients treated with thick and thin membranes [38] and the reported superiority of the peritoneum in clearing 'middle molecules' [4] has not been confirmed [36]. However, there is an alternative explanation for the prolonged survival of patients on regular peritoneal dialysis that fits the hypothesis; at the start of each dialysis about 1 litre of ascitic fluid is removed which has equilibrated slowly with blood since the last dialysis; this makes a sizeable contribution to 'middle molecule' removal [36].

BABB *et al.* [3, 5] studied the dialysance of 'middle molecules' and showed that it was limited by the effective area of the membrane and its thickness, while blood flow rate and dialysate flow rate were much less critical than for small molecules; the dialysance of vitamin B_{12} (MW 1355) plateaued at a blood flow rate or dialysate flow rate of 100 ml/min and was therefore unaffected by changes in these parameters through the range usually employed clinically, unless a decrease in flow rate reduced the effective membrane area through channelling. They designed and tested [15] a regime of low dialysate flow, Kiil dialysis which reduced the removal of urea and creatinine without (predictable) change in 'middle molecule' clearance. The freedom of these patients from neuropathy supported the hypothesis. However, the elevation of plasma urea and creatinine resulting from this regime was not impressive [15, 70] and merely confirmed what many clinicians had long suspected and our present study demonstrates: that plasma urea and creatinine in the range encountered in regular dialysis units are not particularly toxic. An even more convincing demonstration of this fact is provided by the success of dialysate absorption systems [35] in which urea removal may be limited by the low volume of the dialysate and its poor affinity for charcoal [33, 51].

To obtain a more convincing demonstration that middle molecules mattered, CAMBI *et al.* [11–13] made a virtue of necessity; having to crowd patients into an oversubscribed unit, they opted for a short dialysis system, using high blood and dialysate flow and thick membrane – all circumstances favouring selective removal of small molecules. The expected deterioration in nerve conduction did not materialize, and their patient group treated by a regime which favours 'middle molecule' accumulation is now approaching our suggested criterion of 50 patients for 2 years.

There are two methods of removing 'middle molecules' (other than diffusion through the membrane) which must be taken into account when interpreting such trials. Residual renal function of even 1 or 2 ml/min GFR makes a big contribution to overall excretion [60]. Ultrafiltrate contains molecules below the size of inulin in much the same concentration as plasma [1, 63] and makes a further considerable contribution to overall removal. A high ultrafiltration rate through the high resistance coils employed by CAMBI *et al.* [11, 12, 13] can partly explain their unexpectedly good results without discarding the 'middle molecular' hypothesis.

There is now no doubt that unidentified molecules in the molecular weight range 200–2,000, with peaks at about 400 and 1,000 MW [54], accumulate in uraemia and are not reduced by regular haemodialysis [19, 24, 68]. On some current dialysis schedules their accumulation should be 'phenomenal' [69].

There is preliminary evidence of some association between their concentration and some uraemic manifestations [68], but this may prove to be no more meaningful than the long-established similar association with plasma urea and creatinine in the undialysed uraemic. Indeed, the enormous concentration of these substances in some dialysed patients with few complaints argues against their having much toxicity. The substances removed during the first haemodialysis with a very permeable membrane include some which are toxic to tissue cultures and embryonic fish [54], but their relevance to man is uncertain. A study published only in abstract, in which a dialyser with very low permeability to middle molecules was used to treat two patients, did not show any ill effects [83].

We conclude that the 'middle molecular hypothesis' must still be regarded as hypothesis, not as dogma. New regimes of fast dialysis, using high surface area dialysers or very permeable membranes, must be tested to the numbers we have suggested – 50 patients for 2 years – before conclusions are drawn. Before they even get to the clinical trial stage the new regimes must be shown to remove water, potassium, and phosphate in sufficient quantity. So far, high permeability to middle molecules has inevitably been linked with excessive permeability to water, whether high surface area or permeable membrane was employed. Unintentional rapid ultrafiltration was a major hazard of early clinical trials [44]. This risk can now be overcome by use of a closed circuit dialysate pathway [32], and this opens the way for safe, large-scale trials of regimes aimed at selective removal of middle molecules. The rate at which water can be removed from the patient will probably be the limit in shortening dialysis for these trials [27,76], but it may not be the only limit to short dialysis. The dialyser is a greater barrier to the removal of urea and creatinine than the capillary or cell wall [31], and its has been tacitly assumed that this applies to all other molecules in which we are interested; only time will tell whether this assumption is correct.

In the meantime, we need an open mind and large scale controlled trials of short dialysis with very permeable membranes. Whatever they do to the 'middle molecular hypothesis', if they succeed, they should ease the burden of the dialysis patient. 50 fit patients after two years of short dialysis will be more convincing than any amount of mathematical theory or column chromatography.

Acknowledgements

We acknowledge the help of numerous colleagues engaged in the care of these patients, notably Dr. P.R. ULDALL, Dr. R. WILKINSON, Dr. R.W. ELLIOTT, and the Department

of Clinical Biochemistry under the direction of Professor A.L. LATNER, and Dr. A. CASSELLS-SMITH in which the biochemical estimations were performed.

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A Comparison of Short and Long Haemodialysis

79

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Summary

Two groups of patients treated by short (Milan) and long (Newcastle) haemodialysis were compared for incidence of symptoms and biochemical control. Short dialysis corrected urea and creatinine less well but control of potassium and phosphate were similar. The only apparent penalties to be paid by short dialysis patients were a higher incidence of itching, tingling or numbness, impairment of vibratory sense and difficulty in controlling blood pressure. The short dialysis group had higher haemoglobin and less dyspnoea, muscle weakness and dizziness after dialysis.

Introduction

Short haemodialysis prolongs life with a low annual mortality rate and economy in staff (Cambi et al, 1974), but does it exact a penalty in increased morbidity? The best way to answer this question would be a prospective controlled comparison of short and long haemodialysis in a single population allocated at random to each schedule of treatment. We are conducting such a study in Newcastle at the moment but will need up to five years to obtain meaningful results. In the meantime we considered it worthwhile to compare two haemodialysis populations with a similar number of patients of similar age and total duration of dialysis, but in one case (Newcastle) treated for 6-8 hours thrice a week using conventional blood flow (200 ml/min) and the other (Milan) dialysing 4-5 hours thrice a week using a high blood flow (350 ml/min).

MATERIALS AND METHODS

All patients who had been on regular haemodialysis for at least six months in each unit at the time of the study entered the comparison. There were 64 in

Newcastle and 67 in Milan with a mean age of 39 and 41 years respectively. There was a higher proportion of males in Milan: 76%, compared with 53% in Newcastle ($p < 0.01$). The mean total duration of dialysis prior to the study was similar in the two populations: 33 months (6 - 95) in Newcastle and 36 months (6 - 81) in Milan. The aetiology of renal failure was mixed in both centres but there was a higher proportion of patients in Milan with glomerulonephritis as the underlying disease: 58% compared with 39% in Newcastle ($p < 0.05$).

Residual renal function was estimated in each patient by measurement of endogenous creatinine clearance over a long interdialytic period (Molutinovic et al, 1974), in most cases the weekend before their visit. The residual renal function (RRF) was significantly different in the two groups (Table I). However, in only two cases in Newcastle and three in Milan was it between 3-4 mls/min and only one Italian patient had a RRF of 6.2 ml/min.

The only significant differences in the dialysis procedure are summarised in

TABLE I. Residual Renal Function

	NEWCASTLE (n = 64)	MILAN (n = 67)	
Anephric	23%	9%	
None	39%	51%	$p<0.05$
<1 ml per min	20%	10%	
>1 ml per min	17%	30%	

TABLE II. Differences in Dialysis Procedure Between both Populations

	NEWCASTLE	MILAN
Schedule of Dialysis (hours)	6–8 x3/week	4–5 x3/week
Blood flow (ml/min)	200	350
Dialyser	1 m^2 Meltec multipoint	Most of the 1 m^2 commercially available
Preparation of Dialysate using Deionized water	50%	100%
Dialysate calcium (mg/dl)	6.2	7.5
Dialysate glucose (mg/dl)	200	100
Place of Dialysis (home)	59%	40%

Table II. All the other techniques we looked at were similar in both populations. Short dialysis following the regime defined above was started in Milan twenty-four months previously and since then all patients had followed it. Twenty-seven patients had been on short dialysis from the beginning. The rest had previously received twelve hours twice weekly on a Kiil or eight hours twice weekly on the Kolff dialysers.

All patients were asked to complete a symptom questionnaire prior to their visit. Such a questionnaire is routinely used in Newcastle for the follow-up of dialysis patients and it was translated into Italian. The questioning and examination included assessment of muscle power with particular attention to proximal myopathy and clinical examination for peripheral neuropathy. The symptoms compared are shown in Table III. The mean of all pre-dialysis diastolic blood pressure in each patient during the three months prior to their visit was obtained and patients were divided into a 'good control' group whose mean diastolic pressure pre-dialysis was below 100 mm/Hg, a 'moderate hypertension' group with diastolics between 100 and 115 mg/Hg and a 'severe hypertension' group with diastolic blood pressure above 115 mm/Hg. The means of all the pre-dialysis biochemical variables (usually checked once or twice a month) shown in Table III measured during the three months before the study in each patient, were used for comparison.

TABLE III. Variables Studied

Questionnaire and Clinical Examination			Pre-dialysis (means of 3 months)
Dyspnoea	Restlessness	Activity	Urea
Angina	Itching	Sleep	Creatinine
Anorexia	Headaches	Libido	Sodium
Nausea	Dizziness	Sexual function	Potassium
Vomiting	Cramps	Intercourse	Calcium
Tingling	Bleeding tendency	Menstruation	Phosphate
Numbness	Muscle weakness	Neuropathy	Alkaline phosphatase
Twitching	Bone pain	Blood pressure control	Haemoglobin

RESULTS

Table IV shows the comparison of biochemical values. As expected the mean pre-dialysis plasma urea and creatinine were significantly higher in the patients on short dialysis. Nonetheless, the control of some other small molecules, including potassium and phosphate, was adequate. Pre-dialysis serum calcium was higher in

TABLE IV. Biochemical Control in Patients on Short and Long Haemodialysis

Means of predialysis values of the 3 months prior to the study

	LONG	SHORT	
Urea	139	178	mg/dl
Creatinine	11.9	13.6	
Sodium	138	137	mEq/L
Potassium	5.2	4.8	
Calcium	9.9	9.4	mg/dl
Phosphate	5.6	5.0	
Alkaline phosphatase (high)	42%	43%	

All variables in both populations were significantly different (t-test) at at least the 1% level, except the alkaline phosphatase (chi-square).

the Newcastle population in spite of a lower bath calcium, but alkaline phosphatase values were comparable (a Chi-square test was used for normal range for IU was different in the two groups).

Only negative correlations were found between pre-dialysis plasma urea or creatinine and any of the symptoms studied in either population, i.e. patients with no symptoms had higher ureas and creatinines. For instance, the mean urea and creatinine in patients with no itching in Milan were 192 and 15 mg/dl respectively compared with 171 and 13 mg/dl ($p < 0.01$ in both cases) in those complaining of itching. This constitutes further support for our previous finding (Barber et al, 1975; Alvarez-Ude et al, 1975) that within the range studied, plasma urea, creatinine and small molecules which move with them, are not important causes of symptoms. The inclusion of short dialysis patients extends this range of our previous studies: the mean + 2SD for the short dialysis group were plasma urea 247 mg/dl creatinine 19.1 mg/dl.

Since the two populations were not comparable in sex, diagnosis and place of dialysis we analysed the data of each centre separately, to see if any of these factors was associated with any of the other variables in either group. The only significant effect was of sex, which in both groups affected body weight (males higher than females: $p < 0.001$) and consequently plasma creatinine (males higher than females: $p < 0.001$). No effects of primary diagnosis were found.

The place of dialysis affected some biochemical parameters but had little effect on symptoms. Plasma urea was higher ($p < 0.005$) and calcium lower ($p < 0.05$) in hospital patients in Milan while plasma sodium and potassium were higher ($p < 0.01$) in home patients in Newcastle. Newcastle home patients had more nausea and vomiting off dialysis while hospital patients had more insomnia due to the circumstances of dialysis on the night shift. Short dialysis patients at home

had a higher incidence of dyspnoea ($p < 0.05$) and were using antihypertensive drugs more frequently ($p = 0.035$).

Residual renal function was significantly different in the two populations (Table I) but no significant relationship was found between the residual renal function and any biochemical finding or symptom except dyspnoea. The latter was significantly less frequent in patients on short dialysis with a residual renal function 1 ml/min (no patient in this group complained of dyspnoea compared with 36% of the others, $p < 0.0016$). This, coupled with the fact that more home patients on short dialysis had dyspnoea, reflects the greater difficulty in fluid control on short dialysis schedules, particularly in patients who do not pass urine or are dialysed on their own.

The presence or absence of kidneys did have a very clear effect on many variables as shown in Table V.

TABLE V. Effect of Anephric State in Both Populations

NEWCASTLE AND MILAN		ANEPHRIC	NEPHRIC	p
Haemoglobin (mean) g/dl	Newcastle	6.5	8.2	< 0.01
	Milan	6.1	9.2	< 0.001
NEWCASTLE				
Activity	Not working	67%	26%	< 0.01
Bone pain	Present	60%	25%	< 0.02
Sleep	Bad	67%	24%	< 0.01
Sexual function	Depressed or absent	91%	56%	< 0.05
MILAN				
Anorexia	Present	67%	15%	< 0.01
Itching	Severe	50%	7%	< 0.05

We have therefore compared both populations as a whole but whenever significant differences were found, we re-examined the data to see whether they could be explained by the differences in place of dialysis, residual renal function and the presence or absence of kidneys.

Table VI shows the first set of differences between the populations, in the incidence of dyspnoea, control of blood pressure and mean haemoglobin. These differences persisted when anephric patients were compared with the rest. Long dialysis achieved a better blood pressure control and there was no difference in the percentage of patients using antihypertensive drugs in the two centres – 17% in Newcastle and 13% in Milan. However, only one patient in each group was in the 'severe hypertension' range with mean pre-dialysis diastolic blood pressure

TABLE VI. Differences Between Short and Long Dialysis (corrected for anephrics)

	LONG	SHORT	p
Good control of blood pressure	94%	70%	<0.01
Dyspnoea	47%	26%	<0.05
Haemoglobin (g%)	8.2 ± 1.7	9.2 ± 2.0	<0.02

over 115 mm/Hg. In spite of the better blood pressure control, long dialysis patients had more dyspnoea. This presumably reflects their lower haemoglobin which is not entirely accounted for by the higher incidence of anephric patients in Newcastle. In the ranges studied it appears that anaemia is a more important cause of dyspnoea than hypertension; (in fact, the Newcastle patients who complained of dyspnoea had a significantly lower mean haemoglobin: 7.2 g/dl, than those who did not: 8.5 g/dl; $p < 0.01$).

TABLE VII. Differences Between Short and Long Dialysis (corrected for anephrics)

SHORT DIALYSIS WORSE	LONG	SHORT	p
Tingling or numbness	8%	24%	<0.05
Itching	31%	59%	<0.01
Impairment vibratory sensation	11%	27%	<0.05
LONG DIALYSIS WORSE			
Dizziness after dialysis	33%	11%	<0.01
Muscle weakness	29%	8%	<0.01

The other significant differences found between the two schedules of dialysis, after correction for the number of anephrics, are shown in Table VII.

DISCUSSION

The main obstacles to reduction in length of dialysis are control of water balance and the maintenance of pre-dialysis serum potassium and phosphate within adequate limits was obtained. In this study adequate control of potassium and phosphate was obtained in patients on short dialysis. This is no doubt due in part to the

higher blood flow used in Milan and partly to the more rigorous approach to diet in that centre. However, only 27% of the patients in Milan claimed to follow the diet strictly and only 14% in Newcastle made the same claim. In Newcastle where the prescribed diet was smaller, there was a greater tendency for patients to eat more and the converse tendency was seen in Milan. It is therefore likely that the dietary intake of the two populations was not very different. A higher proportion of patients on short dialysis had to use oral phosphate binders. The incidence of bone disease cannot be adequately judged by the incidence of symptoms (Ellis & Peart, 1973) or the level of alkaline phosphatase and we did not compare radiological and histological parameters, so no firm conclusions can be drawn.

Control of blood pressure is more difficult in short dialysis, particularly in home patients and in those passing no urine. Nevertheless, only 25% of the patients on short dialysis showed moderate hypertension and the use of antihypertensive drugs was similar in both groups. The need to control fluid retention in the shorter period of dialysis did not result in a higher incidence of cramps, headaches, hypotensive episodes or dizziness after dialysis in the Milan group. Indeed the last symptom was more frequently seen in long dialysis patients.

The only symptoms encountered more frequently in patients with short dialysis were itching, tingling and numbness of hands and feet and loss or impairment of vibration sense in the toes. We presume that this is an early sign of peripheral neuropathy, but only two patients in Milan showed any other evidence of peripheral neuropathy and no patient was seriously disabled by it. Other published studies of short dialysis have shown no deterioration in neuropathy after more than two years with 1 m^2 dialysers in a large population (D'Amico et al, 1975).

A good reflection of the high degree of rehabilitation obtained with short dialysis is the fact that 79% of the patients in Milan were working full or part-time at the time of the study.

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Open Discussion

KRAMER (Gottingen) There was better control of blood pressure in the group of long dialysers, yet they complained of more dyspnoea. Can you explain this?

ALVAREZ-UDE The patients with dyspnoea had significantly lower haemoglobins than those without dyspnoea.

WALLS (Leicester) There was a greater proportion of female patients on long dialysis than on short dialysis. Were the male patients in Milan receiving androgen therapy? This would give them a high mean haemoglobin. Did you compare the male and female haemoglobins?

ALVAREZ-UDE There was a higher proportion of males in Milan, but they did not receive androgen therapy. There was no difference between males and females for the haemoglobin in either group.

JACOBS (Paris) You did not mention any differences in the patients' diets in the UK and Italy. Can you tell us something about this?

ALVAREZ-UDE Only 14% ate the prescribed diet in Newcastle and only 22% in Milan, so it is quite difficult to draw any conclusions.

David Appleton

Proc. 6th int. Congr. Nephrol., Florence 1975, pp. 650–656 (Karger, Basel 1976)

Residual Symptoms in Patients on Regular Haemodialysis

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Their Relationship to Control of Uraemia and Residual Renal Function

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Regular dialysis (RDT) short enough to allow double shift daytime working in hospital and evening dialysis at home (up to 4 h, thrice weekly) is practised in 3 main styles: *Italian* – with 1-m² dialysers, high blood flow limits accumulation of small molecules, but does not prevent considerable retention of middle molecules [MIGONE *et al.*, 1975]. *French* – the very permeable RP6 dialyser reduces the concentration of larger middle molecules in plasma [FUNCK-BRENTANO *et al.*, 1975], but short dialysis and recirculation cause a considerable rise in small molecules. *American* – high surface area dialysers prevent any rise in middle molecules (except the largest and those held up at cell walls), but permit some rise in small molecules unless blood flow is increased [BABB *et al.*, 1972]. All 3 systems can remove sufficient water, potassium and phosphate (with a little help from phosphate binders) and their relative merits must depend largely on the importance of small and middle molecules in causing uraemic symptoms. Since this is speculative, and likely to remain so for several years, the ideal 'short dialysis schedule' will be found by trial and error. The usual basis for comparison is Kiil dialysis 8–10 h thrice weekly [BARBER *et al.*, 1975], but few centres now employ this regime. Our patients are now treated with the Meltec Multipoint dialyser which is 30% more efficient than the Kiil, for 6–8 h thrice weekly. Biochemical control is essentially unchanged – mean pre-dialysis values for 100 patients on Kiil dialysis 1973: urea 147, creatinine 12.8 mg/dl; mean values for 75 patients on Multipoint dialysers 1975: urea 139, creatinine 12.0 mg/dl. The 'uraemic' symptoms

Table I. Incidence of some 'uraemic' symptoms in two Newcastle surveys

	1973	1975
Nausea	32	31
Muscle weakness	33	31
Cramps	67	65
Restlessness	45	39
Muscle twitching	24	23
Pruritus	39	36
Bone pain	39	29
Menstrual abnormalities	32	50
Reduced libido	55	67
Reduced physical activity	54	51
Bleeding tendency	–	31
Insomnia	–	56

from which our patients suffer, as revealed by our symptom questionnaires, are also unchanged (table I). Multipoint dialysis for 6–8 h thrice weekly is an equivalent for comparison with short dialysis.

We have compared this regime at Newcastle with Italian style short dialysis at Milan [ALVAREZ-UDE *et al.*, 1975]. The present study was carried out on the Newcastle data to decide which of the 'uraemic' symptoms from which our patients continue to suffer can be used as indices of under dialysis. A previous study of 100 patients on Kiil dialysis has been reported [BARBER *et al.*, 1975].

Which Symptoms of Patients on RDT Correlate with Plasma Urea and Creatinine?

Our patients reported on 50 symptoms from which we selected those characteristic of untreated uraemia. In our previous study [BARBER *et al.*, 1975], none of these showed a positive correlation with plasma urea or creatinine pre or post dialysis and we concluded that within the range studied (mean plus 2 SD plasma urea 220, creatinine 17.7 mg/dl) urea, creatinine and small molecules that move with them are not important causes of symptoms. This conclusion has been strengthened by the present study in which we again found no correlation between pre dialysis urea or creatinine and any of the symptoms listed in table I and by a similar study of the 67 patients in Milan on short dialysis [ALVAREZ-UDE *et al.*, 1975].

Table II. Residual renal function in 75 patients on regular haemodialysis at Newcastle

Anephric	15
Nil	18
0–1 ml/min	24
1–3 ml/min	14
3–4 ml/min	4[1]

1 Two dialysed 1 month.

Table III. Guesstimated effect of residual renal function on pre-dialysis concentration of middle molecules and renal hormones

	Pre-D MM of similar size to		Renal hormones, metabolism
	inulin	B_{12}	
Anephric	5 x	2 y	0
GFR 1 ml/min	2.5 x	1.8 y	+
GFR 4 ml/min	x	y	++

Which Symptoms of Patients on RDT Correlate with Retention of Middle Molecules?

A rough estimate of middle molecule concentrations can be made for Newcastle patients. Most of the variation in plasma urea and creatinine between individuals is due to the use of a single size of dialyser for a rather inflexible time period with patients varying in size from under 40 to over 80 kg. We would therefore expect smaller middle molecules to move in parallel with urea and creatinine. However, larger middle molecules should be affected much more by residual renal function. Table II shows the residual renal function (creatinine clearance) in 75 patients studied in 1975. Effect of renal clearance on retention of middle molecules can be predicted, given clearances of the dialyser for molecules of various sizes [KERR *et al.*, 1974], as decribed by POPOVITCH and MONCREIFF [1974]. Our prediction for the effect on our patients is shown in table III. We would not expect much influence on small

Table IV. Predialysis plasma urea, creatinine and weight in 75 patients on multipoint dialysis 3 x 6–8 h

Renal function	Number	Urea, mg/dl	Creatinine, mg/dl	Weight, kg
Anephric	15	140	12.4	57.5
0–1 ml/min	42	136	12.2	58.6
1–4 ml/min	18	137	11.4	63.8

Table V. Symptoms and signs showing no correlation with residual function in 75 patients

Anorexia	Twitching	Menstruation
Nausea	Restlessness	Libido
Vomiting	Cramps	Intercourse
Headache	Itching	Bleeding tendency
Dyspnoea	Insomnia	Neuropathy

and lower middle molecules, and this is confirmed for urea and creatinine in table IV. However, molecules above the size of vitamin B_{12} may be more dependent on renal excretion than dialysis. We therefore analysed our data for 1975 for evidence of any correlation between renal function and symptoms.

Since renal function declines with time on dialysis, it is first necessary to find which symptoms are related to RDT. We therefore compared 39 patients dialysed less than 24 months with 36 dialysed more than 24 months. There was no effect on neuropathy, cramps, twitching, sexual function, itching, anorexia, nausea or vomiting. There was a higher incidence in the longer dialysed patients of weakness (41 versus 21%; $0.05 < p < 0.1$) and bone pain (33 versus 23% NS). Since we have other evidence that these symptoms, and reduced physical activity, progress with time on dialysis in our patients we have analysed them only in the group treated for more than 24 months. There is a striking difference between patients with good residual function and the other two groups, but the numbers are too small to achieve significance. However, the differences between anephric and nephric patients are significant

Table VI. Patients with dialysis encephalopathy at Newcastle 1964–75

Name	Age	Sex	Diagnosis	Syndrome [1]	Months on RDT [2]	BP		At onset, pre-D [4]			Bone histology	Path fractures [5]	Myopathy [6]
						at start	at onset [3]	urea	creat.	Hb			
JG	38	F	Hyp	2	72	190/110	105/70	159	–	8.6	OM	yes	++++
DJ	19	M	Ob.Neph.	1	72	180/115	140/90	115	10.6	6.5	OM	yes	++++
WK	43	M	GN	1	27	210/115	120/70	130	11.8	8.2	–	yes	++++
GA	30	F	CPyelo	1	29	220/130	180/110	90	6.4	5.3	–	yes	++++
TY	37	M	GN	2	96	170/140	115/70	145	14.0	8.2	OM	nil	++
CW	26	M	GN	2	15	180/120	130/60	107	14.1	4.5	OM	yes	+++
GH	31	M	TBK	3	33	180/100	160/90	140	15.5	7.0	OM	yes	+++
TS	42	M	GN	3	60	190/100	170/85	135	13.0	6.5	OM	yes	+++
EY	31	F	CPyelo	3	25	210/130	120/70	130	8.7	5.8	OM	yes	+
AE	34	M	GN	3	30	215/130	130/90	150	10.8	5.8	OM	yes	++
Mean								130	11.7				

1 Syndrome 1 = non-fluent dysphasia; 2 = 1 + dyspraxia, cerebellar ataxia and myoclonus; 3 = 2 + dementia, progressing to death.
2 At onset of syndrome.
3 Mean pre-D BP over last 3 months before onset, rounded to nearest 5 mm.
4 Mean of results over 3 months before onset.
5 Fractures, Looser's zones on X-ray.
6 Clinical myopathy graded on an arbitrary scale.

for bone pain ($p < 0.01$), reduced activity ($p < 0.01$) and almost significant for weakness ($p = 0.06$). Since these symptoms in our patients are usually manifestations of osteomalacia, we suspect that the differences are due to loss of renal endocrine, rather than excretory function, and may well be unrelated to middle molecular accumulation.

None of the other symptoms listed in table V showed any relationship to residual renal function. We conclude that, as far as we can judge in this rather indirect manner, accumulation of middle molecules of all sizes is no more harmful than accumulation of urea and creatinine in the ranges we encounter. The same conclusion has been drawn from our study of the Milan population [ALVAREZ-UDE *et al.*, 1975].

Rarer Manifestations of Uraemia

Neuropathy is rare in our dialysis population. Of the 75 patients studied in 1975 only two had sensory loss in the feet, explained by spina bifida in one. The fear of progressive neuropathy has dominated thinking about short dialysis; our experience adds no substance to these fears and we continue to wonder how often 'uraemic neuropathy' is drug-induced.

Pericarditis was not encountered in these studies, but it does occur about once a year among our 75 patients, sometimes in patients well dialysed by all other criteria [MARINI and HULL, 1972]. However, it was commoner in the past and was then often associated with temporary underdialysis because of shunt problems or increased dialysis requirements during infections. A narrow safety margin against the onset of pericarditis may be one of the penalties of short dialysis.

Encephalopathy is attracting much attention as a measure of adequate dialysis since it can be quantitated. However, the only really devastating manifestation of encephalopathy – 'dialysis dementia' – appears to us to be an unrelated phenomenon which is not due to underdialysis. We have encountered 10 examples in the last 10 years in 384 dialysed patients (table VI). We have found no epidemiological clue to a toxic cause or a role for dialysis depletion. Common features of these patients are a past history of severe hypertension, recent normotension with frequent hypotensive episodes and a very high incidence of osteomalacia and myopathy. Anaemia is common and the exacerbation of symptoms during dialysis, when the oxygen dissociation curve is altering,

suggests a possible role for cerebral hypoxia. Plasma urea and creatinine levels prior to the onset of the syndrome have been slightly lower than the mean for our patients.

Conclusions

Our study of 100 patients on Kiil dialysis and 75 patients on multipoint dialysis (including 27 of the original 100) shows no evidence that any of the symptoms from which they complain is due to retention of urea, creatinine or middle molecules. We therefore have no grounds for preferring one or other of 3 styles of short dialysis other than their cost and convenience. We cannot pick out any symptom which is likely to give warning of underdialysis, but we suspect that an increased incidence of pericarditis will be the first serious sign that dialysis has been shortened too far.

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Glomerulonephritis

International Conference
on Pathogenesis, Pathology and Treatment
Freiburg, June 1976

Edited by Reinhold Kluthe,
Arnold Vogt and Stephan R. Batsford

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A Controlled Trial of Cyclophosphamide in Proliferative Glomerulonephritis

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The treatments recommended for proliferative glomerulonephritis are too numerous to suggest that any of them is very effective. Immunosuppressive have a toxicity well known to nephrologists engaged in transplantation. So we have the classic case for a controlled trial: a killing disease and a toxic treatment of uncertain efficacy. Yet an exponential rise in publications on immunosuppressive therapy for glomerulonephritis between 1963 and 1973 contained only a minuscule proportion of controlled trials (Koeltz et al. 1975).

One reason for this dearth is that few centers have enough patients to run an uncomplicated controlled trial by themselves. They must choose between the tedium of a multicenter trial ending in an anonymous publication and an uncontrolled trial which they can publish under their own name. It is easier to publish the latter if you can extract some positive conclusions, and in the process you may convince yourself as well as the editor. We quote our experience to show how easily one may be misled by a "pilot" trial.

In a gap between MRC trials we conducted an uncontrolled trial of cyclophosphamide and concluded that it had benefited the majority of patients with proliferative glomerulonephritis (Table 1). However, we criticized our own data and did not publish them. We had not had an obligatory observation period before treatment to exclude patients recovering spontaneously. One of the five who became protein-free was in fact suffering from subacute bacterial endocarditis treated with antibiotics and another may well have had recovering poststreptococcal nephritis. We had not decided in advance

the period over which we would judge success or failure; in selecting them retrospectively, we had left ourselves an opportunity for introducing bias. The same criticisms can be leveled at many published uncontrolled trials.

Table 1 Uncontrolled trial of cyclophosphamide in 30 patients with proliferative glomerulonephritis, analyzed retrospectively

Complete remission – no proteinuria	5	
Improvement	8	
Renal function stabilized	5	
	–	
		60%
Progression to renal failure	7	
Continued deterioration	3	
Death – septicemia	1	
Death – nasopharangeal carcinoma	1	
	–	
		40%

We therefore planned a controlled trial of cyclophosphamide. Since the MRC was just starting a national trial of indomethacin, the study was confined to our center. Newcastle had contributed about 20% of the patients in previous MRC trials, so we anticipated an entry of only 50–60 patients over 3 years, not sufficient to rely on simple randomization to provide well-matched treatment and control groups in so variable a "disease". We had first to subdivide the patients on the basis of characteristics which had a major effect on prognosis. At the time we were engaged in a retrospective study of proliferative glomerulonephritis and we used the preliminary results of that study to select the important parameters. The full study will be published elsewhere. A brief description is given here.

Retrospective Study of Proliferative Glomerulonephritis

Between 1960 and 1970, 1357 renal biopsies were performed at the Royal Victoria Infirmary. Among these were 218 biopsies performed on patients with adult-onset (aged over 14) proliferative glomerulonephritis. From these we selected the first 100 in datal order on whom we had sufficient biopsy material for quantitative analysis and whom we had followed for at least 2 years, or until death or dialysis if earlier; 79 of the patients had a 5-year follow-up. One of us (J.S.) abstracted the clinical information on each patient. Only those items which were available on most patients were utilized, eliminating many observations which we would have available on patients seen in the 1970's. The renal biopsies were assessed semi-quantitatively by A.R.M.; no specimen with less than 6 glomeruli was utilized. 3–5 μm sections stained with H & E, PAS, acid picromallory and silver methenamine were examined without knowledge of the clinical features. Clinical parameters used were those measured at the time of biopsy or within 1 week, except the blood pressure for which the pretreatment value was used. Fifty-two histologic measurements, covering glomerular, tubular, interstitial and vascular pathology were carried out and composite scores of total glomerular, tubular, interstitial and vascular damage were calculated.

The data, in original form or after log transformation, were first examined by t-tests to find the individual parameters which predicted survival at 2 and 5 years. The more

promising data were then analyzed by computer to find a discriminant function which best predicted survival.

Results

Only a brief resume is presented. Of the patients, 49 'survived'* at 2 years, 29% of those at risk survived for 5 years. This reflects the severity of disease referred to us in the early 1960's. Of the *clinical characteristics,* age, sex and, to our surprise, blood pressure had little prognostic value. Quantitative proteinuria was not significantly correlated with outcome at 2 years but just significantly correlated with survival at 5 years (heavier proteinuria → poorer survival).

The ASOT titer, graded in 7 grades from normal to very high titer, correlated strongly ($p < 0.005$) with survival at 2 years, the higher ASOTs giving the best prognosis. The two other clinical parameters with good predictive value were initial renal function, judged by plasma urea or creatinine, and mode of presentation (Table 2).

Table 2 Retrospective study of proliferative glomerulonephritis. Effect of mode of presentation on outcome at 2 years

Presentation	Number	% survival at 2 years
Acute renal failure	28	11%
Nephrotic syndrome	25	56%
Asymptomatic proteinuria	22	73%
Recurrent hematuria	9	100%

The *histological parameters* which gave the best prediction of 2-year survival were the percentage of glomeruli surrounded by epithelial crescents ($p < 0.001$), and the scores of total tubular damage ($p < 0.005$). Histologic measurements were better predictors of outcome than the clinical measurements available to us in this study, though this no doubt reflects the incompleteness of some of the clinical data.

Various *discriminant functions* incorporating only histologic or mixed clinical and histologic parameters were calculated. The best so far obtained is:

$z = 0.335 + 0.750$ ASOT score $- 0.0306$ Crescent score $- 0.244$ Total interstitial damage score.

A 'z' below -2.8 indicates less than 5% chance of surviving 2 years, a score of over $+2.8$ indicates a better than 95% chance of survival.

A discriminant function of the type shown above would be of great value in pairing candidates for a prospective trial if the patients were already available at the start. However, we had insufficient patients already attending our clinics, so had to abandon this approach; new patients were admitted to the trial throughout a 3-year period.

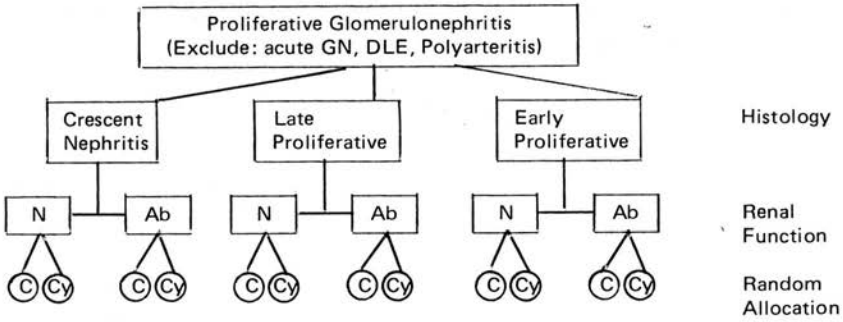
We therefore used the findings in Table 3 as indicated. The scheme of allocation we finally employed is shown in Table 4.

*Throughout this paper "survival" indicates survival on the patient's own renal function. Regular dialysis/transplant are statistical "deaths".

Table 3 Clinical and histologic parameters giving good prediction of outcome at 2 years in the retrospective survey; manner in which they influenced the planning of the prospective trial

Clinical Features	
Initial renal function	Patients subdivided before randomization
Mode of presentation (Table 2)	ARF excluded by preliminary observation period. Patients with isolated hematuria excluded from trial (treatment not justified)
ASO titer	Patients with any clinical or histologic suspicion of poststreptococcal nephritis excluded from trial
Histologic features	
Crescents	'Crescent nephritis' (over 50% glomeruli affected) treated as a separate subgroup
Total interstitial damage)) Total tubular damage)	Biopsies classified as 'early' or 'late', mainly on the basis of these criteria, and patients in these categories allocated separately

Table 4 Newcastle cyclophosphamide trial, scheme of allocation



Prospective Controlled Trial of Cyclophosphamide

A preliminary report of the trial, with follow-up to 2 years, has been published (Koeltz et al. 1975). The final follow-up to 3 years has now been completed and is reported here.

Methods

Patients with a clinical and histologic diagnosis of proliferative glomerulonephritis were followed for 3 months during which time recovering acute glomerulonephritis, disseminated lupus and polyarteritis were carefully excluded. If at the end of this period they still had active nephritis, as shown by proteinuria of at least 1 g per 24 hours and a typical urinary deposit, they were classified in the histologic and clinical subgroups shown in Table 4.

The differentiation of "early" and "late" proliferative glomerulonephritis was made on criteria agreed by the three pathologists reporting renal biopsies, using the features which were incorporated in our scores of total interstitial and tubular damage in the retrospective study. Although the criteria are arbitrary the recognition of "early" and "late" lesions proved reproducible between pathologists and when the same pathologist

re-examined the biopsies "blind" after an interval of 3 years. The poorer prognosis of a "late" lesion (i.e. one showing considerable scarring of the kidney) was confirmed in this trial.

Patients in each subgroup were allocated to treatment or control observations by drawing an envelope from a pack, after they had given informed consent.

The cyclophosphamide group received this drug orally for 12 months or until death or dialysis if earlier. The initial dose was 1 mg/kg/day and it was increased by small increments at fortnightly intervals until the white cell count fell below 4,000/mm^3. If the count fell below 3,000, the dose was reduced. All patients in both groups were given symptomatic treatment for edema and hypertension. No patients received corticosteroids or other immunosuppressive drugs.

The observations carried out on patients in both groups are shown in Table 5. They were performed at the following intervals: (0 = time of allocation)

Months	−3 − 0	Monthly	13 − 18 2-monthly
	1 − 12	Monthly	18 − 24 3-monthly
			24 − 36 6-monthly

Results were averaged for 3-monthly periods until the end of the treatment year, 6-monthly thereafter, for purposes of analysis.

Table 5 Observations carried out at each assessment on all patients

Clinical	
	Assessment of edema
	Blood pressure
	Examination of fundi
	Urine microscopy
Laboratory	
	Quantitative proteinuria (24 hour)
	Plasma urea, creatinine, Na, K, Cl and HCO_3
	Creatinine clearance (24 hour)
	Serum total proteins, albumin, electrophoresis
	Serum cholesterol
	Serum C_3
	Urine culture
Additional tests on patients in treatment group	
Every 2 weeks:	Hb, WBC (Platelet and differential counts if required)
	Question re side effects
	Adjust dose of cyclophosphamide

Results

The size of the various subgroups has been reported previously (Koeltz et al. 1975). Only 3 were large enough for random allocation to work well. Nonetheless, our allocation scheme worked better than we had dared to hope (Table 6). We ended up with equal-sized groups, with an even spread of the different grades of renal histology and of normal and abnormal renal function (judged by serum creatinine). The only two clinical and histologic subgroups we could recognize – mesangiocapillary nephritis and Schönlein-Henoch syndrome – were also well divided between the groups.

Table 6 Controlled trial of cyclophosphamide. Allocation of patients

	Control	Cyclophosphamide
Total	26	27
Early proliferative	18	19
Late proliferative	6	6
Crescent nephritis	1	2
Normal renal function	8	10
Impaired renal function	18	17
Mesangiocapillary	2	3
Schönlein-Henoch	4	5

Follow-up to 3 years has not altered in any way the conclusions in our preliminary report. We found no difference between treated and control patients, whether considered together or as histologic or clinical subgroups, in any of the parameters listed in Table 5.

Did we postpone or prevent death? (Table 7)

The answer is clearly "No". Death was at least as common in the treatment group and occurred after the same interval as in the control patients.

Table 7 Controlled trial of cyclophosphamide. Outcome at 3 years

	Control	Cyclophosphamide
Death from:		
Uremia	1	2
Infection	1	1
Myocardial infarction	1	0
Meningioma	0	1
Dialysis/transplant	5	6
	8	10
Mean time (months) from start of treatment/control observation to dialysis/transplant/death	14	14

Did the renal function of survivors benefit?

Again the answer is 'No'. The results at 3 years were unchanged from our preliminary study at 2 years. Mean serum creatinine rose and fell in both groups as patients went into renal failure and left the group but at no point was there a significant between groups, or even a trend in favor of the treated patients.

Did patients with 'unscarred' kidneys do any better on treatment?

No. When the patients with early proliferative glomerulonephritis are separated out, the conclusions are unaltered.

Did cyclophosphamide give symptomatic relief by reducing proteinuria?

Again "No".

On the other hand, cyclophosphamide did produce side-effects, which were discussed

in our preliminary report (Koeltz et al. 1975) and are summarized in Table 8. The most important was gonadal failure, shown in the women by the onset of amenorrhea and menopausal symptoms and presumably occurring in the men but not eliciting complaint since cyclophosphamide appears to affect fertility rather than potency (Buchanan et al. 1975). The problem of gonadal failure is discussed below.

Table 8 Controlled trial of cyclophosphamide. Drug side-effects in 27 patients

Alopecia	2/27
Nausea/diarrhea	1/27
Leukopenia (2000/mm^3)	0/27
Cystitis	0/27
Amenorrhea	5/7

Conclusions

Cyclophosphamide in the dosage given to the type of patient selected (fairly severe, since mortality was 35% in 3 years) conferred no benefit and had side-effects.

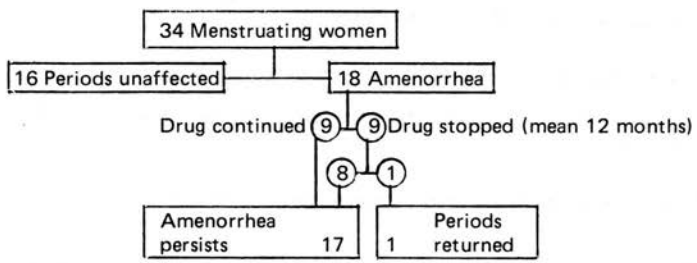
Gonadal Failure in Patients on Cyclophosphamide

Amenorrhea was not a serious disadvantage to the patients in this trial. They had, as a group, severe disease with a poor life expectation and none was anxious to increase her family. Menopausal symptoms were controlled with estrogen replacement, where necessary. However, the problem becomes more serious if cyclophosphamide, or any other alkylating agent, is to be used in future trials or earlier disease, with a good prognosis, in young adults.

In Table 9 we summarize the incidence of amenorrhea in 36 women treated during our peak enthusiasm for the drug in renal disease and previously reported briefly (Uldall et al. 1972). Only one of the women in whom the drug was discontinued regained her periods. One other has recently begun to menstruate again, after a gap of about 3 years in which she was treated with azathioprine for SLE. However, her plasma LH and FSH remain elevated and we suspect that she is still infertile. One reason for the high incidence of permanent ovarian damage in our experience was undoubtedly our policy of continuing the drug beyond the onset of amenorrhea

Table 9 Amenorrhea complicating oral cyclophosphamide for glomerulonephritis, minimal change and SLE

Dose → WBC < 4000/mm^3 (40–140 mg/day)



(Table 10), either because we felt the severity of the disease justified it or because the association had not then been reported. In most cases amenorrhea did not appear until after 5 months' therapy with cyclophosphamide orally, but a few patients developed this complication within 1 month of starting treatment.

Table 10 Details of 18 women developing amenorrhea during cyclophosphamide therapy

	Median	Range
Age (years)	33	19–43
Duration of therapy (months)		
to amenorrhea	6	0–13*
Total	20	5–33

*0,0,1,1,5,5,5,6,6,6,7,7,10,12,13

Clearly any future trials that include this drug or other alkylating agents should include a warning about gonadal side-effects before informed consent is obtained and should allow the clinician to stop therapy promptly at the first sign of menstrual irregularity.

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Artificial Organs
10(3):226–235, Raven Press, New York
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Particle Spallation Induced by Blood Pumps in Hemodialysis Tubing Sets

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Abstract: The repeated flexion and compression of pump segments by the rollers of peristaltic pumps results in cracking and abrasion of the inner surfaces of the pump segment, leading to shedding of particles into the extracorporeal circuit. A series of studies to assess the rate of particle release from silicone rubber, polyvinyl chloride (PVC), and Pivipol, a coextruded polyurethane-coated PVC tubing, when these materials were used with blood pumps of the type found in hemodialysis units, was undertaken. The studies show that with all tubing/pump combinations there is an overall increase in the total number of particles released, but an analysis of the particle size distribution indicates that the majority of the particles are <16 μm in diameter. The rate of increase may be reduced, however, by decreasing the occlusion pressure. **Key Words:** Hemodialysis—Spallation—Silicone.

Peristaltic roller pumps are widely used in extracorporeal circulation, and although a number of different designs are available, their mode of operation is similar. A segment of compressible tubing, normally of silicone or polyvinyl chloride (PVC), is inserted into a curved roller track and held in position by clips. The rollers of the pump rotate within this track and compress the tubing. This compression generates the flow in the circuit since it creates a pressure gradient across the insert and forces the fluid along and out of the pump segment. After each passage of the rollers the tubing segment resumes its original shape, fresh fluid is drawn into it to refill it, and the process is repeated.

Peristaltic roller pumps in clinical use have two or three rollers on the pump shaft, and their speed of rotation is potentiometrically controlled. The position of the rollers relative to the tubing may be fixed or adjustable by means of a Vernier scale whereas in some newer designs the rollers are spring-loaded to enable optimum occlusion to be achieved even in the presence of dimensional variations of the pump segment that may exist between different batches of tubing.

The repeated flexion and compression of the segment by the rollers and its passage over the pump insert result in cracking and abrasion of the internal surfaces of the pump segment. This leads to shedding of particles into the extracorporeal circuit. Spallation of particles in extracorporeal circuits used in cardiac surgery is a well-recognised complication of the procedure (1–3). Hemodialysis, unlike cardiac surgery, is performed on a routine, three-times-a-week basis, with each treatment lasting between 4 and 6 h depending on the patient's size and clinical requirements.

Postmortem specimens from patients receiving long-term dialysis have demonstrated the presence of refractile particles. These particles have been identified as silicone originating from the extracorporeal circuit used in the treatment (4–7). As a consequence of these findings, we have undertaken a series of studies to assess the rate of particle release, and to analyse the factors influencing particle release in blood tubing sets used for haemodialysis.

Received August 1985; revised February 1986.
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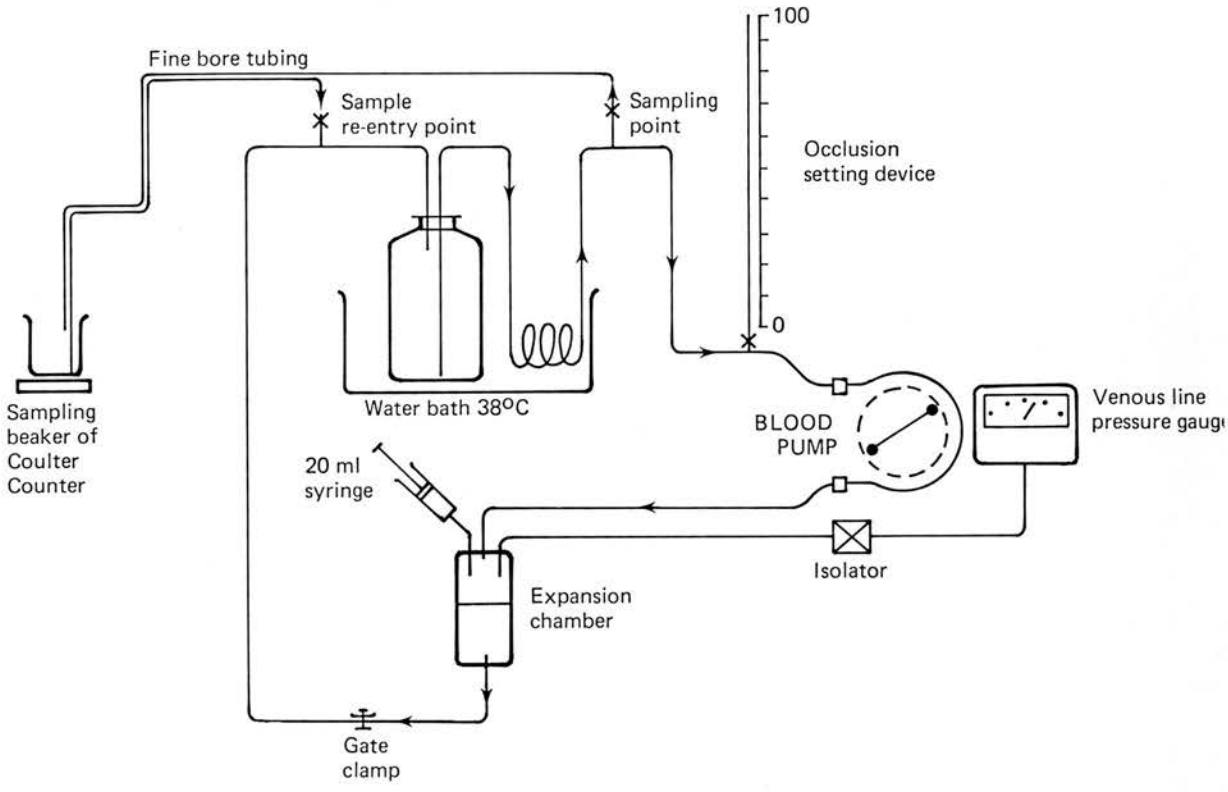


FIG. 1. Experimental circuit for in vitro particle release determination.

MATERIALS AND METHODS

Blood pumps

Three commonly used blood pumps of the type found in hemodialysis units were studied: the Watson Marlow MHRE (Watson Marlow Ltd., Falmouth, U.K.), a three-roller pump in which the roller position is fixed relative to a curved roller track, but which may be moved to alter the occlusion; the Bellco BL 705N (Bellco SpA, Mirandola, Italy), a twin-roller pump in which the position of the rollers is adjusted relative to the roller track by

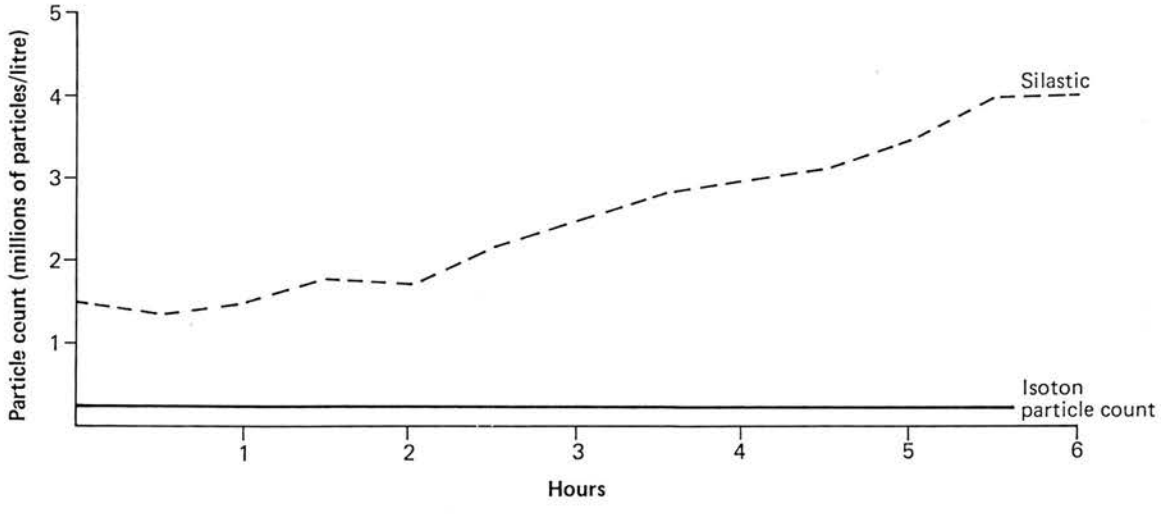


FIG. 2. Total particle count based on the mean of observations during four runs for Watson Marlow MHRE pump during 6 h. Total number of particles in the 2–80 μm range are shown. Silastic, silicone rubber.

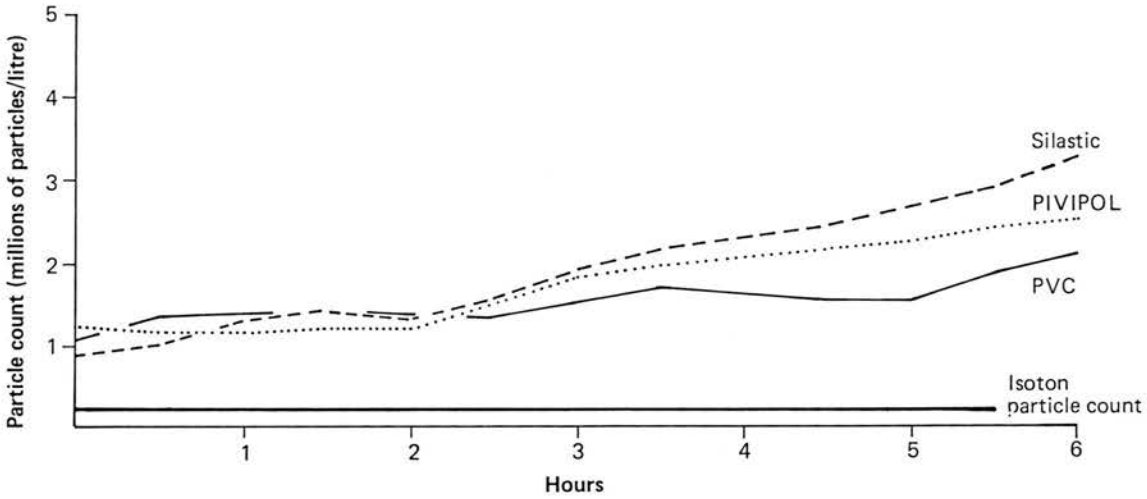


FIG. 3. Total particle count based on the mean of observations during four runs for Bellco blood pump during 6 h. Total number of particles in the 2–80 μm range are shown. Silastic, silicone rubber; PVC, polyvinyl chloride.

means of Vernier screws; and the Fresenius blood pump (Fresenius AG, Oberursel/Taunus, F.R.G.), a twin-roller pump in which the rollers are spring-loaded. This last pump was studied with two spring tensions: 96N (Fresenius A) and 115N (Fresenius B).

Blood tubing materials

Three blood tubing materials were selected for use with the above pumps: medical grade silicone rubber (Extracorporeal SA, Waremme, Belgium, and Fresenius AG, Bad Homburg, Germany); PVC (Bellco SpA, Mirandola, Italy, and Fresenius AG) and a co-extruded polyurethane-coated PVC tubing (Pivipol, Bellco SpA). All tubings used in the studies came from single-production batches, and their internal diameters ranged from 6.3 to 8 mm while their wall thicknesses varied from 1.7 to 2.1 mm.

Experimental technique

The experimental circuit shown in Fig. 1 was used. In order that the degree of occlusion in each of the studies was comparable, the pump roller occlusions were set by the use of a technique in which the working occlusion was defined as the point where a 100-cm column of water supported by the pressure of the occlusion fell at a constant rate of 2.5 cm/min. To obtain the working occlusion with the Bellco pump, the Vernier screw was adjusted for each experiment, whereas with the Watson Marlow pump, the position of the race track on its angled base plate was moved relative to the rollers. No such adjustments were possible in the Fresenius pumps, which worked on a fixed occlusion.

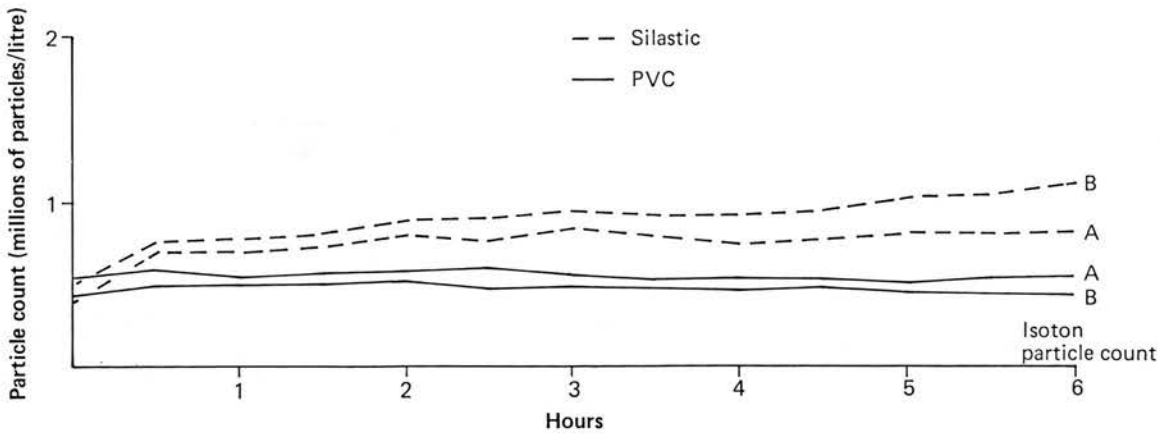


FIG. 4. Total particle count based on the mean of observations during four runs for Fresenius blood pump during 6 h. Total number of particles in the 2–80 μm range are shown. Silastic, silicone rubber; PVC, polyvinyl chloride.

TABLE 1. *Total number of particles between 2 and 80 μm in diameter (count/L × 10^3) generated during tubing/pump studies*

Material	Pump	Time (h)											
		0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5
Silicone rubber	Watson Marlow	1,587	1,362	1,500	1,812	1,338	2,238	2,575	2,832	—	3,113	3,450	4,000
	Bellco	937	1,050	1,313	1,475	1,338	1,638	1,975	2,200	—	2,525	2,763	3,000
	Fresenius A	469	701	706	722	789	767	846	—	712	751	791	798
	Fresenius B	518	743	796	816	928	904	945	932	960	982	1,085	1,047
PVC	Bellco	1,188	1,375	1,387	1,462	1,425	1,425	1,638	1,788	—	1,600	1,662	1,963
	Fresenius A	563	602	556	578	590	603	—	523	529	545	512	540
	Fresenius B	450	507	503	498	533	474	498	—	466	517	472	455
Pivipol	Bellco	1,250	1,462	1,450	1,513	1,450	1,550	1,850	2,038	—	2,287	2,375	2,512

Average coefficient of variation for the four observations was 27%.

The extent of spallation observed with the tubing pump combinations over a 6-h period was assessed. Silicone rubber tubing was used with all three pumps, the use of PVC was confined to the Bellco and Fresenius pumps, and Pivipol tubing was used only with the Bellco pump. Owing to the low torque generated by the Watson Marlow MHRE pump, it was not possible to use the pump with either PVC or Pivipol tubing.

Assessment of the number of particles with diameters between 2 and 80 μm released during the study period was by the use of a Coulter Tall particles counter (Coulter Electronics, Luton, Bedfordshire, U.K.). The instrument was connected to the flow circuit by fine-bore tubing, as shown in Fig. 1. The tubes were arranged in such a way that the pressure gradient across the pump insert could be utilized to deliver and return fluid from the circuit to the sample chamber. The flow through these tubes was 28 ml/min at the time of sampling, and the sampling volume used on each occasion was 2 ml. Sedimentation in the samples drawn for analysis was prevented by a mechanical stirrer in the sampling chamber.

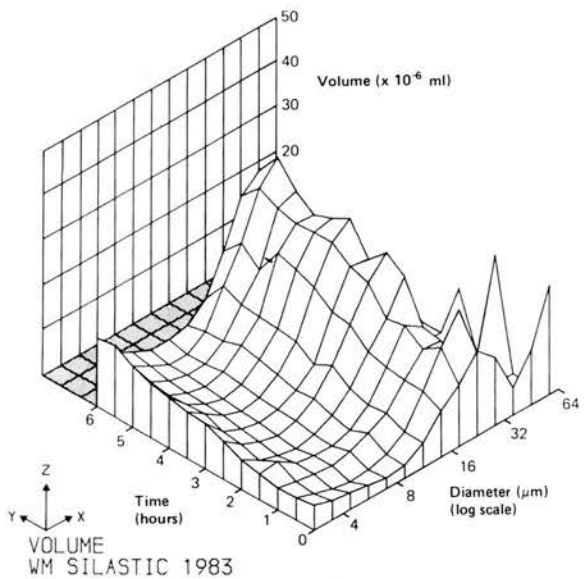


FIG. 5. Isometric plot of total volume of particles observed using the Watson Marlow MHRE pump. Mean of observations during four runs is shown. Silastic, silicone rubber.

The volume of the extracorporeal circuit was 300 ml, and a flow of 200 ml was maintained throughout the studies to simulate clinically used flow rates. Withdrawal and return of the fluid to the reservoir was at two different levels to ensure thorough mixing in the reservoir, particularly of larger particles during the experimental study. In addition, a pressure of 100 mm Hg in the venous drip chamber was maintained.

Before each series of measurements, the circuit was flushed with 2 L Isoton 11, a buffered filtered electrolyte solution, which was also used as the recirculated fluid.

A series of four studies for each of the pump insert material and pump combinations was performed after the technique has been established; each study lasted 6 h, and samples were taken for particle analysis every 30 min during the study. The volume lost by sampling was not returned to the circuit, and results were corrected to account for the changes in concentration resulting from volume changes induced by the sampling. Calibration of the Coulter counter was performed prior to each run by the use of a latex suspension in distilled water containing particles of known diameter.

Each sample taken was recorded in terms of the number of particles of a given size per liter, and hence the total and percentage volume of particles of a given size.

A supplementary series of studies was performed to determine the particle counts of fresh electrolyte

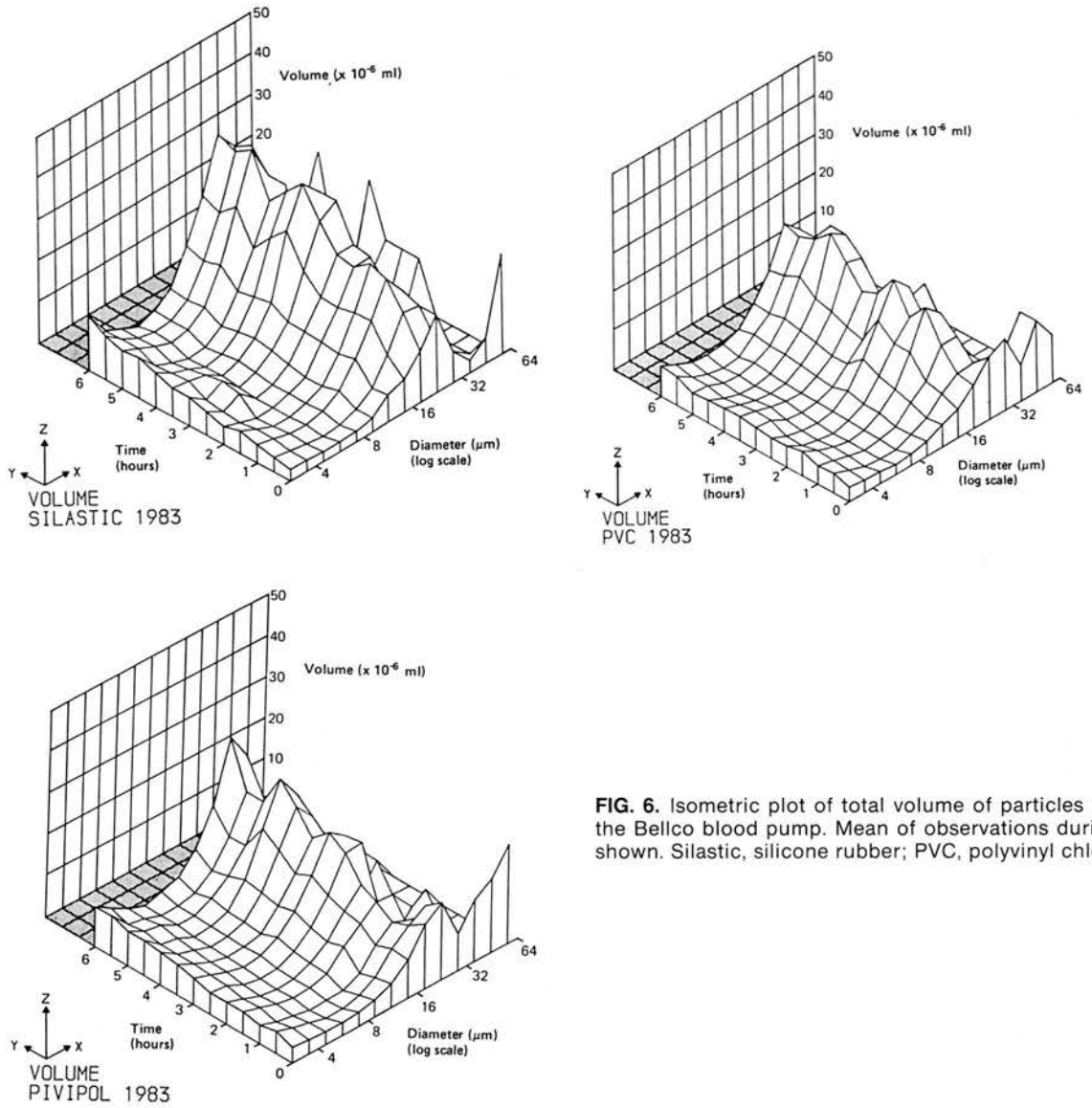


FIG. 6. Isometric plot of total volume of particles observed using the Bellco blood pump. Mean of observations during four runs is shown. Silastic, silicone rubber; PVC, polyvinyl chloride.

solution (200,000–250,000 particles/L). Airborne dust contamination from the surroundings was assessed by exposure of Isoton 11 to the laboratory environment for a 5-h period, with samples being removed every hour. No discernible contamination was present, but a small number of particles in the 20–50-μm range was observed. In order to minimise the influence of these particles on the overall results, the experimental circuit and sampling chamber were isolated from the surrounding laboratory environment.

Since the samples drawn for analysis were mechanically stirred, the possibility of spurious counts from microbubbles introduced by the stirring process could not be excluded. To minimise this effect, a series of studies in which the influence of stirrer speed on particle count was undertaken. These studies demonstrated that at maximum stirrer speed setting there was a significant contribution from this source (2,000,000 particles/L); however, at the speeds used for the experiments, the contribution from this source was small (20,000 particles/L).

At the termination of each experiment, samples of tubing were removed from the pump insert for scanning electron microscopy.

RESULTS

The particle counts observed for each of the pump/material combinations, based on the mean of observations during four runs, are shown in Figs. 2–4 and summarized in Table 1.

Figures 5–7 show the isometric plots of the total volume of particles for each blood-pump/tubing combination based on the mean of observations during four runs. The x axis in these plots represents the diameter of the particles on a logarithmic scale, the y axis represents time, and the z axis represents the volume per milliliter of fluid, made up by particles of a given diameter at a given time. Thus, the frequency polygons for total volume as a function of time at a given diameter and as a function of diameter at a given time are shown.

Clinical interest is focused on particles >16 μm diameter, and a further analysis of the data obtained for such particles was performed. Since the series of studies performed in 1983 (Watson Marlow and Bellco pumps) exhibit different features from the 1984 series (Fresenius A and B), the results have been analysed separately.

Analysis of covariance was performed to establish the relationship of number of particles (>16-μm diameter) with elapsed time of pumping and types of pump and material. For the 1983 series, number of particles increased linearly with time, at a rate not significantly affected by pump or mate-

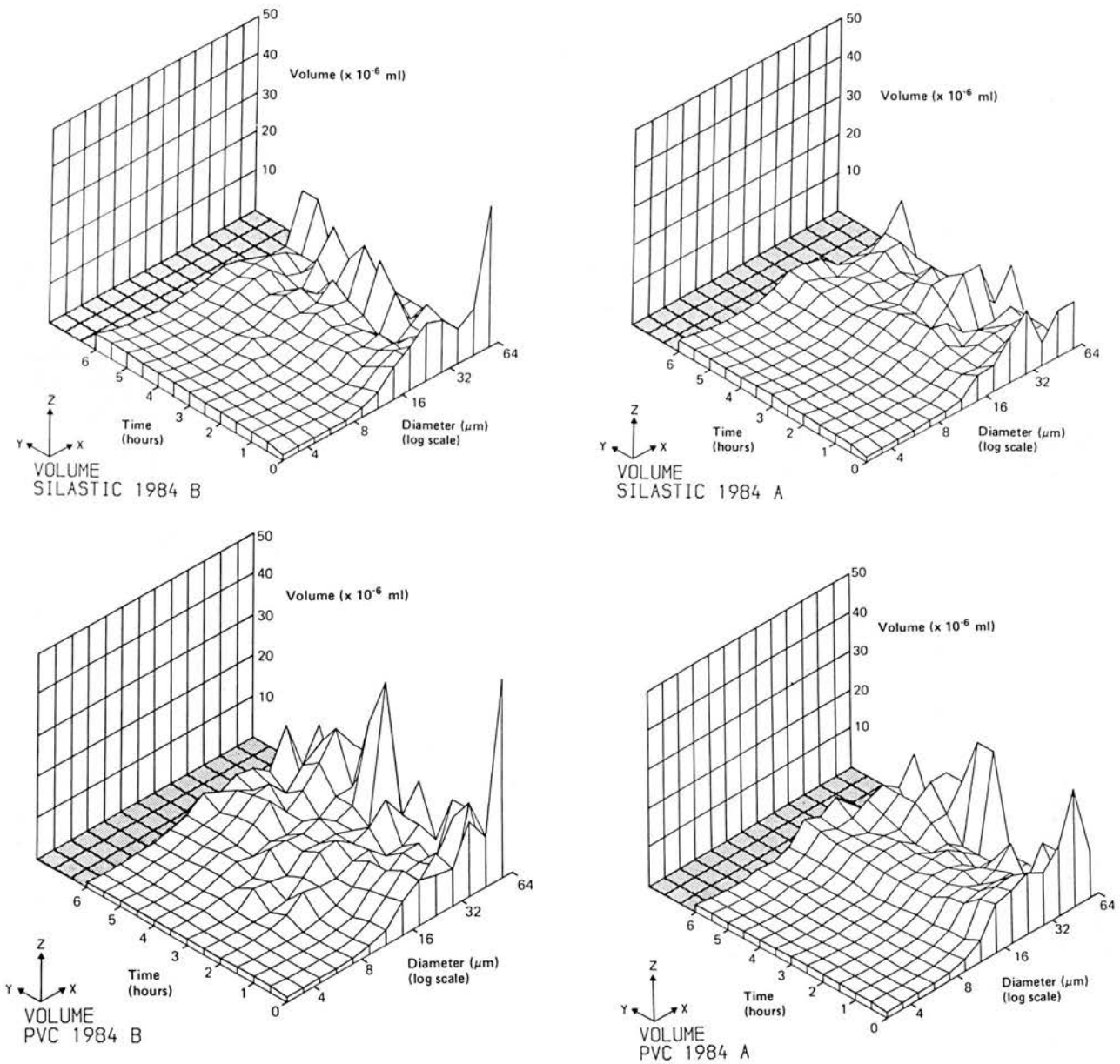


FIG. 7. Isometric plot of total volume of particles observed using the Fresenius blood pump. Mean of observations during four runs is shown. Silastic, silicone rubber; PVC, polyvinyl chloride.

TABLE 2. *Relationship between number of particles (>16 μm) released per ml and elapsed time of pumping (h)*

Pump	Material	Particles released/ml
Watson Marlow[a]	Silicone rubber	18.9 + 2.7 × time
Bellco[a]	Silicone rubber	22.5 + 2.7 × time
Fresenius A[b]	Silicone rubber	3.8 − 0.35 × time
Fresenius B	Silicone rubber	
Bellco[a]	PVC	14.4 + 2.7 × time
Fresenius A[b]	PVC	6.7 − 0.35 × time
Fresenius B[b]	PVC	
Bellco[a]	Pivipol	13.4 + 2.7 × time

[a] 1983 experimental series.
[b] 1984 experimental series.

rial. However, there were highly significant ($p < 0.001$) differences between materials, there being more particles after a given time with silicone rubber than with PVC or Pivipol. Results are summarised in Table 2.

A similar analysis using the 1984 data for the Fresenius pumps showed a decrease with time for particles >16 μm diameter. The materials were significantly different with PVC worse than silicone rubber. There was, however, no difference between different tensions, a finding contrary to that of Bommer et al. (8), who demonstrated that reduction of occlusion pressure effectively reduced spallation in silicone rubber.

Macroscopic examination of the pump inserts after 6 h of pumping showed a permanent flattening

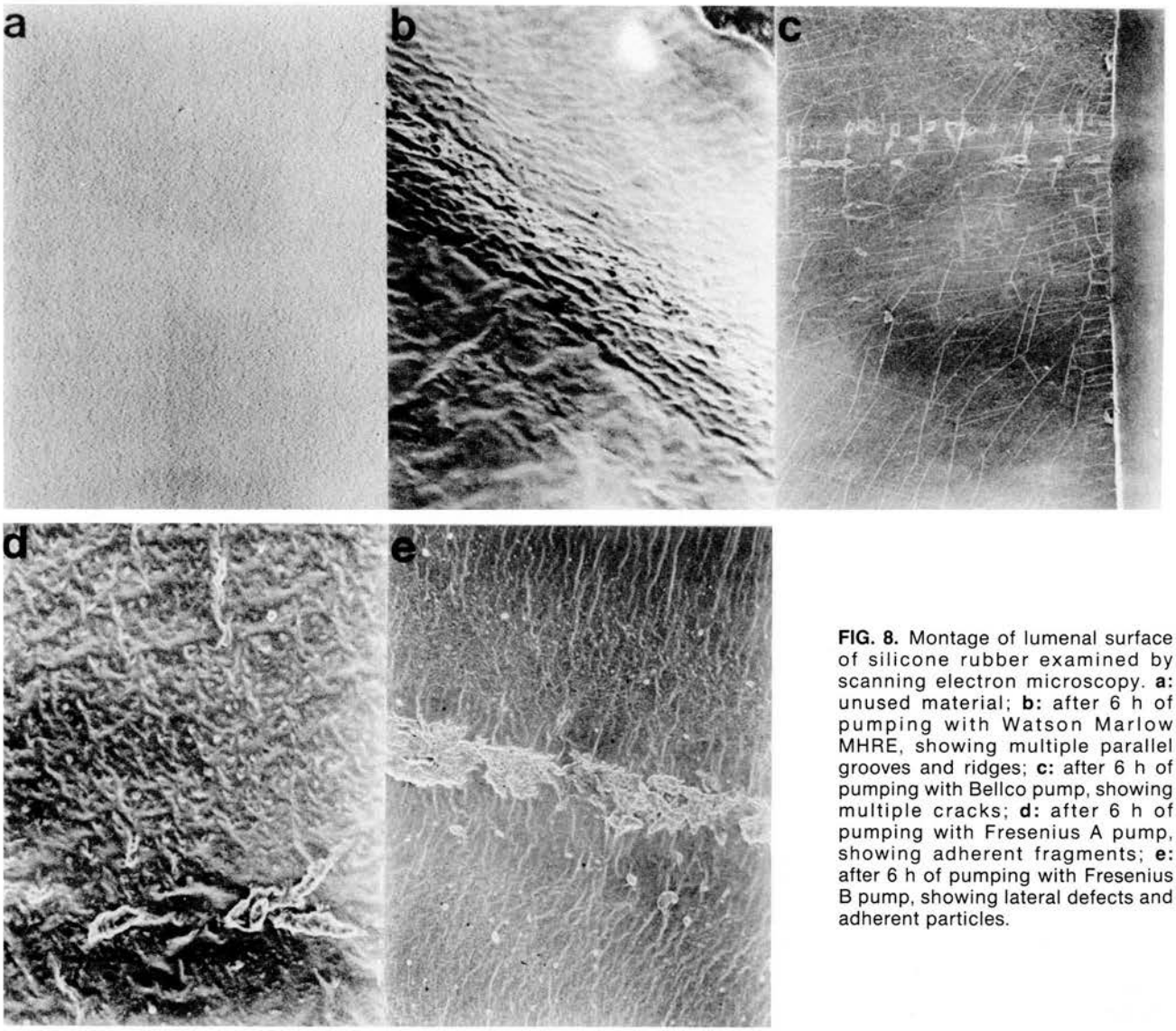
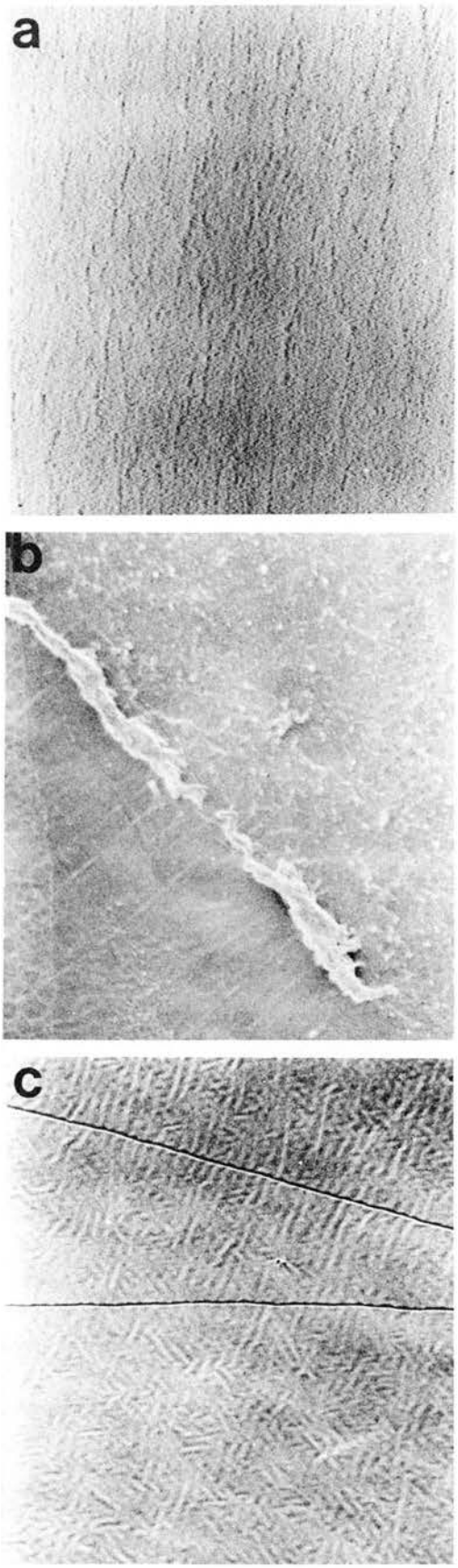


FIG. 8. Montage of lumenal surface of silicone rubber examined by scanning electron microscopy. **a:** unused material; **b:** after 6 h of pumping with Watson Marlow MHRE, showing multiple parallel grooves and ridges; **c:** after 6 h of pumping with Bellco pump, showing multiple cracks; **d:** after 6 h of pumping with Fresenius A pump, showing adherent fragments; **e:** after 6 h of pumping with Fresenius B pump, showing lateral defects and adherent particles.



of the tube to an oval form with a loss of translucency for PVC and Pivipol and an increased capacity of the normally opaque silicone rubber. At the point of maximum flexure, external ridges were present.

Scanning electron microscopy was performed on the materials before and after use. Figure 8 shows the montage of lumenal surface of silicone rubber, and Figs. 9 and 10 show similar montages for PVC and Pivipol.

Unused tubing was comparable in appearance with occasional superficial imperfections. In contrast, used tubing demonstrated lateral cracks at the point of maximum compression together with superficial fractures. Furthermore, adherent particles were also noted on the surface of the material originating from the fractures.

DISCUSSION AND CONCLUSION

Our studies have demonstrated an overall increase in the total number of particles with time for the Watson Marlow and Bellco pumps when used with PVC, silicone rubber, and Pivipol. The mean patterns illustrated were observed consistently within each set of experiments, and the average coefficient of variation was 27%. The largest number of particles released using silicone rubber with these two pumps probably occurred early in the experiment. Use of PVC and Pivipol with the Bellco pump resulted in a reduction in the total number of particles observed, but we failed to distinguish between PVC and Pivipol, which suggests that the polyurethane lining of the latter offers only a limited protection from structural damage induced by the action of the pump rollers. The second series of studies using the Fresenius pumps demonstrated no overall increase in the number of particles with time.

Initial counts in all experimental studies were found to be higher than can be explained by the particle count in the Isoton solution, this difference being attributable to the shedding of particles during the setting up and equilibriating of the experimental circuit.

Analysis of the data in terms of particle size distribution shows that the majority of the particles are <16 μm in diameter. Particles greater than this show an increase with time, except in the case of

FIG. 9. Montage of lumenal surface of polyvinyl chloride (PVC) examined by scanning electron microscopy. **a:** unused material; **b:** after 6 h of pumping with Bellco pump, showing adherent fragments; **c:** after 6 h of pumping with Fresenius A pump, showing multiple cracks.

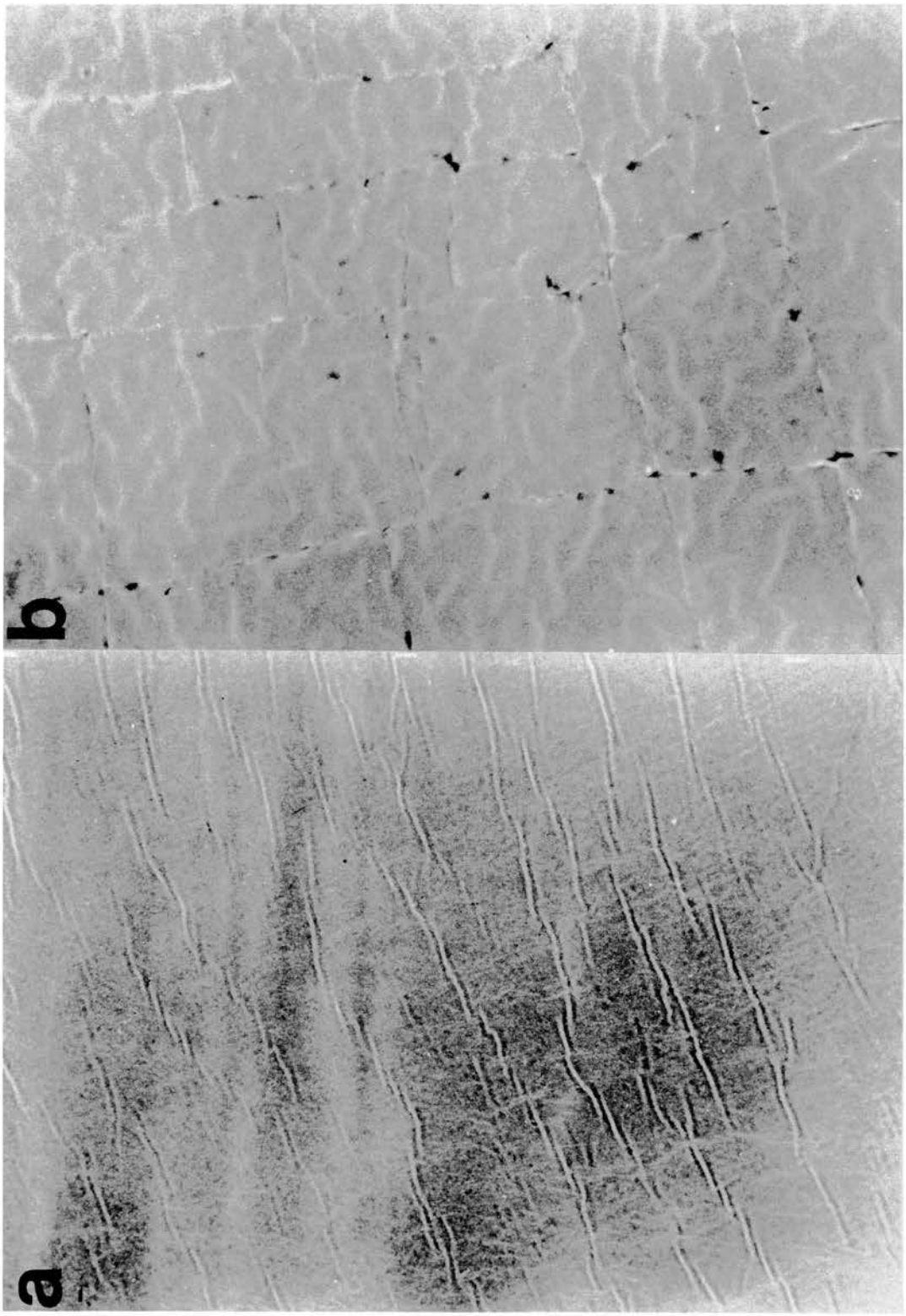


FIG. 10. Montage of lumenal surface of Pivipol examined by scanning electron microscopy. **a:** unused material; **b:** after 6 h of pumping with Bellco pump, showing superficial cracking.

the Fresenius pump, where a paradoxical decrease was observed. Two possible explanations are offered. First, clumping of the particles may have occurred, causing their size to fall outside the limit of measurement; or second, since the rotational speed of this pump is higher than for the others studied, the large particles may have been broken up into smaller particles, the size of which lay outside the detection limits.

Scanning electron microscopy has demonstrated the presence of cracks in the material near the line of flexure formed by the repeated compression and relaxation of the tubing caused by the passage of the rollers. These cracks are the subject of complex forces during pumping, which result from the compression perpendicular to the direction of roller movement, as well as compression ahead of the roller and tensile stress after it. These forces cause the cracks to open and close while the walls may also slide against each other—actions that lead to the release of particles. In addition, the inner surfaces of the pump insert are subject to frictional drag forces occurring as a result of the internal surfaces of the insert being brought together between a moving roller and a fixed pump housing.

The greatest damage in the pump insert appears to be at the edges of the pump insert rather than between the opposed surfaces at the midpoint of the tubing, suggested that frictional drag forces are of secondary importance in the release of particles but may contribute to the breakdown of larger particles and account for the observed decrease in large particles noted with the Fresenius pump.

Boretos and Wagner (3) demonstrated that spallation may be reduced if the tubing wall thickness is decreased, whereas Bommer et al. (8) investigated the role of occlusion pressure and similarly showed that decreased occlusion pressure resulted in decreased spallation.

Optimal occlusion of the pump insert is highly desirable, since excessive occlusion causes increased damage to the pump insert material, it being necessary for the tubing to be completely compressed during pumping to enable flow to occur. Improvements in roller design and alteration of tubing shape may lead to a reduction in damage to the lateral margins of the pump insert and hence the minimisation of spallation.

Acknowledgment: We should like to express our gratitude to Bellco SpA and Fresenius AG for financial and practical support of this study.

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Nephrol Dial Transplant (1988) 3: 91–94
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Nephrology
Dialysis
Transplantation

Data Analysis in Nephrology

83

Analysis of Data in Nephrology
I. Choosing the Correct Statistical Test: Dichotomous Variables

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Abstract. This is the first of six articles describing how to choose the correct statistical test to look for relationships between two variables. All examples relate to clinical or laboratory aspects of nephrology. In this article the overall strategy is outlined, and examples are given of analyses of data where each variable can take only two values.

Key words: Dichotomous variables; Nephrology: Statistics

Introduction

Many clinicians and other medical research workers have attended courses in statistics, or have taught themselves from textbooks to analyse their data. Using pocket calculators or computer packages they can carry out statistical calculations. Sometimes they have access to medical statisticians for advice or encouragement, but often, particularly for smaller analyses, they are left to their own devices. This is not due to unwillingness on the part of the statisticians to be involved with small analyses, but they are too often so busy analysing large trials and surveys with which they have been associated since the design stage, and to which they owe first use of their time, that they are unable to be of any great help.

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It is clear that what often causes most difficulty to the non-statistician is not carrying out the calculations, or even interpreting the results, but choosing the appropriate test in the first place. (It may in fact be that the real difficulty is in designing a proper study, at which point all the methods of analysis would have been decided along with all the other vital decisions such as what is the hypothesis being investigated, how many subjects will be used, who they are, what laboratory methods will be used, and so on, but we shall restrict ourselves here to statistical tests).

This article therefore tries to set out a framework for deciding on the appropriate method to use when testing the statistical significance of a relationship between two variables, and presents some examples of a particularly frequently encountered situation. Subsequent articles will provide an analysis of further examples, all of which will deal with data from studies in clinical or laboratory nephrology. The data are based on real, published articles, but have been in most cases adapted, mainly to make the amount of data easier to handle. This is not to advocate small trials, but is done purely to keep these articles from becoming too long. No attempt will be made to discuss more complicated methods than those for eliciting the relationship between two variables, as simple rules would be inappropriate for other than simple cases.

References to the sources of the original data have not been given, as it is not the author's intention directly or indirectly to criticise published work where the method of analysis was not what is suggested here. The author hopes that where colleagues recognise themselves as sources of data or study designs they will accept his thanks and apologies. A warning is in order: data which lead to

Table 1. Tests to be discussed in this series of articles, classified according to the types of variables involved

		Response variable	
		Dichotomous	Continuous
Explanatory variable	Dichotomous	χ^2 test for 2×2 table Fisher's exact test Odds-ratio test McNemar's test	t-test paired t-test Mann–Whitney U test Wilcoxon signed-rank test log-rank test
	Other discrete	χ^2 test for $2 \times k$ table linear trend in proportions linear trend in logits	one-way analysis of variance randomised blocks Kruskall–Wallis test Friedman test
	Continuous	logistic–linear model	linear regression non-linear regression rank correlation

statistically significant results in this series of papers should not be taken as evidence of real associations, and non-significant results may arise merely because sample sizes have been reduced.

The Framework

We shall assume that it is possible to regard one of the variables as a response variable (length of survival, presence of side-effects, creatinine clearance, etc.) and one as an explanatory variable (treatment given, age, sex, etc.). A particular variable may in different situations be a response variable or an explanatory variable; for example although creatinine clearance could be a response variable varying with treatment given, it could also be used as an explanatory variable if the response variable was survival time. This then is the first decision; if you cannot decide which is the response variable and which the explanatory, the rules which follow will be of no help.

Secondly it is necessary to decide with which types of variables we are dealing. We shall assume that the response variable is either *dichotomous* or *continuous*. A dichotomous variable can have only two values: a subject either has Raynaud's phenomenon or not; 2 years from diagnosis he is either alive or not. A continuous variable is one measured on a continuous scale such that if two individuals appear to be exactly equal that is only because of the imprecision of the measuring equipment. Strictly speaking, counts (e.g. white blood cell count, number of episodes of vomiting) are not continuous, but they may usually be treated as such. It makes intuitive sense to talk of average WBC, and that is a good guideline for regarding a variable as continuous. It is important to look at each variable in terms of the individual: a dichotomous variable for an individual leads to an overall rate in a group of individuals (60% of patients having cramps, for instance), but this rate, though apparently continuous, is only the summary of the values of a dichotomous variable.

A further possibility is allowed for the explanatory variable: it may be *discrete* while not being dichotomous. In other words it may consist of a set of three or more categories (different modes of presentation, different dialysates, etc.) and these categories may be ordered (social class, mild/moderate/severe disease). Table 1 shows the possible combinations we shall consider and the possible tests within these combination of variable types. Other tests exist for the situations we shall cover, and for other situations, but they are not as easily amenable to simple rules. We shall provide 19 examples of data, and it is hoped that the reader will try to analyse each set of data before looking at the analyses given.

In discussing the examples we shall in general not give formulae for the methods used. These are available from many textbooks, and we shall give references in particular to Armitage and Berry [1]. The present articles are not intended to be an introduction to statistics: it is hoped they will amplify what the reader already knows.

Dichotomous Variables

When the outcome of a clinical trial involving two treatments is dichotomous (success of failure), or when one is investigating a possible association between the presence or absence of a disease and the possession of a 'risk' factor, it is usually appropriate to construct a 2×2 contingency table. However, there are several possible ways to analyse such a table, not all of which may be equally appropriate in a particular instance.

Example 1

Two dialysis treatment schedules were compared in a study of vascular side-effects. Of 40 subjects on treatment A 16 had symptoms of Raynaud's phenomenon, compared to 12 out of 45 on treatment B.

Both the response variable (Raynaud's phenomenon) and the explanatory variable (treatment schedule) are dichotomous. The data can be arranged in the form of a 2×2 contingency table:

		Treatment		
		A	B	Total
Raynaud's phenomenon	Yes	16	12	28
	No	24	33	57
	Total	40	45	85

A χ^2 test can be applied (Armitage and Berry [1], p. 125), giving $\chi^2 = 1.70$ with 1 degree of freedom: not significant. Alternatively, Yates' corrected χ^2 can be calculated (Armitage and Berry, p. 129), giving $\chi^2 = 1.15$ with 1 degree of freedom: not significant. Which of these tests you use is not very important as long as you are consistent. If you use the corrected χ^2 test in any publication it is helpful to readers to say that you have done so. Make sure you know which calculation your computer package performs.

Example 2

Two dialysis treatment schedules were compared in a study of vascular side-effects. Of ten subjects on treatment A, five had symptoms of Raynaud's phenomenon, compared to one out of eight on treatment B.

Why should this be different from Example 1? The data can again be expressed as a contingency table:

		Treatment		
		A	B	Total
Raynaud's phenomenon	Yes	5	1	6
	No	5	7	12
	Total	10	8	18

The numbers are too small for the χ^2 test to be applied. As a working rule, if any of the frequencies are smaller than 5 or the total number of subjects is less than 50 use Fisher's exact test (Armitage and Berry, p. 129). The tail probability for the table is given by different packages as either 0.24 (doubling the single-tail probability to obtain a two-tailed test) or 0.15 (adding all probabilities less than or equal to the one for the observed table). Again, make sure you know which version you are using and make sure your readers know too.

Example 3

Of 54 glomerulonephritis patients with severe glomerular damage 14 survived on their own renal function for 2 years; when glomerular damage was mild 30 out of 37 did so.

There would be nothing wrong in analysing by a χ^2 test the contingency table formed by these figures:

		Glomerular damage		
		Severe	Mild	Total
Loss of renal function by 2 years	Yes	40	7	47
	No	14	30	44
	Total	54	37	91

However, it is probably better in this case to estimate the relative risk of loss of renal function for patients with severe rather than mild glomerular damage, instead of testing for an association. We know that there will be an association: what we want to do is quantify it. This is often the case when the response variable is either a disease (present or absent) or an outcome (favourable or unfavourable) and the explanatory variable is a risk factor (present or absent). The calculations are less familiar than those so far given, so will be presented here. They are quite straightforward (Armitage and Berry, p. 456).

For the table:

		Risk factor	
		Present	Absent
Outcome	Unfavourable	a	b
	Favourable	c	d

calculate the log-odds-ratio $r = \log_e (ad/bc)$
and its standard error $s = \sqrt{\{(1/a)+(1/b)+(1/c)+(1/d)\}}$.

Then the best estimate of the odds ratio (approximate relative risk) is given by ad/bc and 95% confidence limits by $\exp(r \pm 1.96s)$. For the table in the example our estimate is 12.2 and our 95% confidence limits 4.4 to 34.1. Since the limits do not include 1 we know also that the outcome is significantly related to the risk factor, but more importantly we have quantified the relationship and the precision of our knowlege about it.

Example 4

In a cross-over trial involving 50 patients, two dialysis treatment schedules were compared. Ten patients complained of vomiting on both schedules, 30 on neither, nine on treatment A only, and one on treatment B only.

Again this may be expressed as a 2×2 table:

		Nausea on treatment A	
		Yes	No
Nausea on treatment B	Yes	10	9
	No	1	30

In Example 3 the χ^2 test was not carried out, though its use could be defended. *Here it would be quite wrong.* How do we know? There are two points to look out for: firstly, the trial had a cross-over design: this means that there is something extra to take into account in the analysis. Secondly, the variables which make up the rows and columns of the table are not now the response and explanatory variables, so we are not testing for an association between the variables which define the table. The appropriate significance test involves looking at the ten subjects who responded differently to the two treatments and testing whether they were divided significantly unevenly between the treatments. Patients who had nausea with both A and B, or who had no nausea with either A or B, provide no information about which is the more nauseous and are omitted from the test. A binomial test (or a sign test, or McNemar's test) is called for (Armitage and Berry, p. 120). Here the probability is 0.021. It may seem strange that this test actually compares the proportions of patients (38% and 22%) vomiting on each treatment, but it is so. It would be possible from the data to form the table:

		Treatment		
		A	B	Total
Nausea	Yes	19	11	30
	No	31	39	70
	Total	50	50	100

However this is wrong. There were only 50 subjects so the overall total should not be 100: these 100 results are not independent observations, as they are matched in pairs.

Final remarks

The examples in this article have covered the four statistical methods in the top left hand corner of Table 1. Subsequent articles will deal with the rest of the table.

Acknowledgements. I am very grateful to Dr A. R. Morley for reading the draft of the articles in this series and for his valuable comments. My thanks also to Mrs S. Knaggs for helping me to prepare the typescript.

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Nephrol Dial Transplant (1988) 2: 225–227
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Nephrology Dialysis Transplantation

Data Analysis in Nephrology

Analysis of Data in Nephrology II. When to Use the t-Test

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Abstract. This is the second of a series of six articles describing how to choose the most appropriate statistical method to test for relationships between two variables. When the response variable is continuous and the explanatory variable is dichotomous, the t-test is often used. The article indicates when this is really appropriate and when the test should be modified or replaced by another.

Key words: Nephrology; Non-parametric tests; Statistics; t-Test

Introduction

When the response variable in an experiment or a clinical trial is continuous, and the explanatory variable is dichotomous (treatment A or treatment B, symptom present or absent) it is usual to think of a relationship between the variables expressing itself as a difference between the two sets of values of the response variable corresponding to the two values of the explanatory variable. If the two sets of observations are each Normally distributed with the same variance the t-test is obviously appropriate, but problems arise when the assumptions of the t-test cannot be met. This article tries to show by means of examples when it is still satisfactory to use the t-test, and what to do when the t-test is inappropriate. The differences in analysis brought about by different experimental designs are also exemplified. (Example numbers continue from the first article in the series [1].)

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Example 5

Fourteen patients had their proteinuria measured, and were also assessed for presence of IgG immune complexes, with the following results:

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Proteinuria (g/24 h)	0.6	0.3	1.2	1.5	1.7	0.3	2.3	2.9	0.5	2.9	3.6	1.8	1.5	2.0
IgG immune complexes	−	+	+	+	−	+	+	+	+	−	+	+	−	+

It is not absolutely clear that proteinuria is the response variable and the presence or absence of IgG immune complexes the explanatory one, but there is no real reason why we should not approach the data from this point of view. Let us therefore separate the proteinuria results into the two samples, and put them in ascending order to get a better idea of the distributions.

IgG complexes−	0.6	1.5	1.7	2.9						
IgG complexes+	0.3	0.3	0.5	1.2	1.5	1.8	2.0	2.9	2.9	3.6

With so few observations per sample it is not worth carrying out a formal test of Normality, but there is no reason to suppose that there is any great departure from it, and the t-test (Armitage and Berry [2], p. 106) is certainly robust enough to cope with any that there is. For these particular data results are so far short of significance ($t = 0.038$ with 12 degrees of freedom: $P = 0.97$) that it makes little difference what test is applied, but when the

sample sizes are as discrepant, as here, and one sample is so small that only a very imprecise estimate of the underlying variance can be obtained, it is as well to carry out the t-test without assuming that the variances are equal. Most statistical packages provide Welch's adaptation of the t-test, which eliminates this assumption (Armitage and Berry, p. 109).

Example 6

Sixteen patients randomised to one or other of two haemodialysis schedules had their blood urea measured immediately after dialysis. Results (in mg/100 ml) were as follows:

Schedule 1	20	34	36	41	50	81	99	121
Schedule 2	45	54	73	98	110	116	160	196

Like so many other concentrations the blood urea distribution is not Normal: it might be difficult to be certain of this from these data, but for schedule 1 for instance, the highest observation is one and a half times further from the mean (60.2) than is the lowest observation. The data for schedule 2 are less obviously skew (the mean is 106.5) but its standard deviation is 52 compared to 36 for schedule 1.

These indications, that the distribution is skew and that the standard deviation increases with the mean, taken with previous knowledge of the shape of blood urea distributions in other publications, should lead us to using logarithms. It does not matter whether these logarithms are common (base 10) or natural (base e), but it should be stated in the description of the analysis if any of the logarithms are given, e.g. the means and standard deviations of the two samples. However it is not usually necessary to do this: to describe the samples use logarithms to find the geometric mean (the exponential function of the mean of the natural logarithms) and 95% limits for the observations (the exponential function of {the mean $\pm$t standard deviations of the natural logarithms}).

For the given data we have:

	Geometric mean	95% limits
Schedule 1	51.4	30.8–85.6
Schedule 2	95.6	62.5–146.1

The t-value for comparison of geometric means = 2.209 with 14 d.f. In this case we have legitimately obtained a significant result which we would not have got without transforming the data. This will by no means always happen: indeed the t-test often gives quite similar *P* values whether or not logarithms are used, but for data such as urea, creatinine, alkaline phosphatase, and many others, it is well to become used to dealing with logarithms.

Example 7

A group of 20 patients with impaired renal function were assayed for urine lead and the results subdivided by sex.

	Urine lead (μmoles)										
Males	3	5	5	8	10	15	20	22	33	47	211
Females	4	6	7	10	16	19	29	38	69		

The analysis here depends on how one approaches the data. If sex is really the only variable to take into account our attitude may well be different from when there is another variable such as severity of disease to think of. The main problem is the observation of 211 in the male group. We cannot assume Normality: we might use logarithms of the observations, and we probably would if there were other possible explanatory variables. If there is an obvious reason for the outlying observation we might report it and otherwise exclude it from our analysis, but that should always be a last resort. The remaining option is to use a non-parametric test: either the Wilcoxon rank-sum test or the Mann–Whitney U test (Armitage and Berry, p. 411). Both give identical results. In this case the advantage of the non-parametric approach is that the extreme observation is included in the analysis but its actual value is ignored. It is accepted that it is the highest observation, but it is not allowed artificially to inflate the mean of the male group or the variance. There are no fixed rules about when to use a non-parametric test, but it is wise to consider a Normalising transformation first. Here, however, an indication of the strangeness of the data is that, while the mean and the geometric mean for the males are higher than those for the females, the median for the males is actually lower than for the females. Of course this inconsistency is also a reflection of the lack of a significant difference between the groups, but such aspects of the data give us insights into it.

The result of carrying out a Mann–Whitney test is a U-statistic of 50.5, an approximate Normal variable of 0.076, and a corresponding *P* value of 0.94.

Example 8

Eight hypertensive dialysis patients undergoing a new treatment had their plasma aldosterone measured (in pmol/l) before treatment and after 1 month.

Patient		1	2	3	4	5	6	7	8
Plasma aldosterone	Before treatment	165	480	236	259	225	355	366	198
	After 1 month	177	603	276	290	219	341	456	210

In this experiment each patient is 'used as his own control'. We therefore need a paired t-test (Armitage and Berry, p. 104), possibly after a transformation, or the non-parametric analogue, Wilcoxon's signed rank test (Armitage and Berry, p. 410). It is not important what the distribution of plasma aldosterone looks like, either before or after treatment: what matters is the distribution of the differences. Let us calculate them and put them in ascending order to appreciate their distribution better.

Patient	6	5	1	8	4	3	7	2
Difference (rise)	−14	−6	12	12	31	40	90	123

The distribution is rather skew (to the right): let us look at the differences in (natural) logarithms.

Patient	6	5	8	1	4	3	7	2
Difference (rise)	−0.027	−0.040	0.059	0.070	0.113	0.157	0.220	0.228

This is rather better, which indicates that there is a tendency for the response variable to increase by a constant *multiple* rather than by a constant amount. We can give an estimate of that multiple as the exponential function of the mean difference in logarithms (1.10), and we can give 95% confidence limits for it as the exponential function of the mean ±t standard errors of the mean difference in logarithms. These come out as 1.01 to 1.20.

Since these limits do not include unity the change is significant at the 5% level, but it is because we can quantify the change in this way, rather than merely perform a significance test, that parametric methods are to be preferred to non-parametric ones whenever possible. Notice, incidentally, that although non-parametric methods are sometimes called 'distribution-free' they do not necessarily give the same results irrespective of whether logarithms are used. In the example just given, taking logarithms before carrying out Wilcoxon's signed-rank test gives a significant result which is not quite obtained without the transformation.

Final Remarks

There are no hard and fast rules about how to analyse data such as have been presented in this paper. Very often the alternative methods will give comparable results, and you may indicate in a publication that this is so. Do *not* choose the method of analysis purely on the basis of its ability to give significant results. Try to be consistent throughout a publication. When in doubt, take logarithms.

One particular kind of continuous variable, namely survival time, should not be treated in the ways described in this article. It will be discussed in more detail in the next article.

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Nephrol Dial Transplant (1988) 3: 340–344
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Data Analysis in Nephrology

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Analysis of Data in Nephrology III. Survival Analysis and Analysis of Variance

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Abstract. In this article discussion is continued of the case where the response variable is continuous and the explanatory variable is discrete. Two particular situations are covered: the response variable survival time and the explanatory variable dichotomous: and the response variable continuous (but not survival time) and the explanatory variable discrete (but not dichotomous).

Key words: Analysis of variance; Nephrology; Statistics; Survival analysis

Introduction

In the previous article in this series we dealt with the case of testing for a relationship between a continuous response variable and a dichotomous explanatory variable. One response variable which must be handled differently (i.e. neither the t-test nor its non-parametric analogue are statisfactory) is survival time, or time to the occurrence of an event of any sort, if death or the event is not certain to occur within a very short time. Were we to deal with mean survival time we would need to wait until all subjects had died: even with a Mann–Whitney U test we would need to wait until all the subjects in one of the two groups had died. The correct method of analysis involves the log–rank test. This allows for the fact that at the time of analysis many of the subjects may still be alive, or they may have been lost to follow-up. In either case their times of death are known as 'censored' observations. We shall not give details of how to carry out the log-rank test, though an example of its use will be given. Further details may be obtained in an article by Peto et al [1].

This article also extends the examples dealt with in the previous article, by considering how to assess the relationship between a continuous variable and a discrete variable which has more than two categories. No fundamentally new ideas are involved, except when the categories have an inherent ordering.

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The Log-rank Test

The log-rank test is a strange name for a test in which the calculation of the resulting χ^2 statistic involves neither logarithms nor the assigning of ranks to the survival times. It can be used when there is a discrete nuisance variable (e.g. sex) involved as well as the principal variable of interest (e.g. treatment) but we shall not consider that extension here.

Example 9

Forty patients with nephrotic syndrome were studied in order to examine the relationship between duration to

terminal renal failure and various biochemical measurements. The results given below are for serum albumin, it being considered 'low' if less than 2 mg/100 ml, and 'high' otherwise. Data relate to the time when the first subject had been followed for 10 years.

Patient	Serum albumin	Alive or dead	Follow-up (months)
1	high	dead	85
2	high	alive	119
3	high	alive	118
4	high	dead	87
5	high	dead	26
6	low	dead	20
7	low	dead	23
8	low	dead	60
9	low	alive	95
10	high	alive	87
11	low	dead	14
12	high	alive	78
13	low	alive	78
14	high	dead	56
15	high	alive	76
16	low	dead	53
17	low	dead	44
18	low	dead	37
19	high	dead	5
20	high	dead	60
21	high	alive	59
22	high	alive	54
23	high	dead	17
24	high	alive	53
25	high	alive	47
26	low	alive	44
27	low	dead	22
28	high	alive	38
29	high	alive	31
30	low	alive	30
31	low	alive	23
32	high	alive	20
33	low	alive	17
34	high	dead	16
35	high	alive	13
36	high	alive	12
37	high	alive	11
38	low	alive	9
39	high	alive	6
40	low	alive	5

The survival curves for the two groups of patients are shown in Fig. 1. The log-rank test yields a χ^2 value of 2.178 with 1 degree of freedom. Of course, serum albumin is not naturally a discrete variable: it is possible that making it discrete has resulted in a loss of information, which in turn has led to the loss of a significant result. The difficulty is that relating survival time to a continuous variable necessitates building parametric models and is a much more complex procedure. The log–rank test is a relatively simple non-parametric approach. If you are comparing two treatments it is the method to use. It does not just compare two survival rates at a particular time: it compares the whole survival processes until that time. For explanatory variables which have more than two categories or are continuous, consult your statistician.

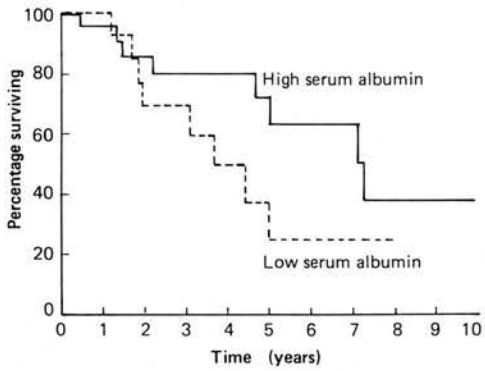

Fig. 1. Survival curves showing duration to terminal renal failure in two groups of patients with nephrotic syndrome (Example 9).

Analysis of Variance

With continuous response variables other than survival time it is not difficult to test for a relationship with a discrete variable with several categories. As with the t-test, the discrete variable is used to form groups whose distributions are compared. The parametric approach uses analysis of variance, the exact version of which depends on whether the observations are all independent, or whether they are matched in 'blocks', e.g. several observations on each patient. There is also facility for taking into account inherent ordering in the discrete variable. The non-parametric analogues are the Kruskall–Wallis and Friedmann tests, in which case ordering of the categories is not taken into account.

Example 10

Three groups of uninephrectomised dogs had their ammonia production measured. Two groups had previously been rendered acidotic, by hydrochloric or lactic acid. Data are given in μmoles per 100 ml GFR.

Control	HCl	Lactic acid
61	75	70
58	81	93
67	101	62
79	86	61
51	69	87
43	49	68
52		
37		

Let us first summarise the data.

	Normal	HCl	Lactic acid
Sample size	8	6	6
Mean	53.0	76.8	73.5
Standard deviation	9.6	17.5	13.4

If we analyse the data as they stand we obtain an analysis of variance (Armitage and Berry [2], p. 186) with an F-statistic of 6.63 on 2 and 17 degrees of freedom, leading to $P=0.0075$. There is therefore a significant difference between the treatment groups, and it is perfectly clear from the means that this arises between the treated groups (taken together) and the control. There is no reason to assume that this interpretation is wrong: the data show no signs of non-Normality, and the standard deviations are not significantly different (Bartlett's test gives $\chi^2=1.92$ with 2 degrees of freedom—Armitage and Berry, p. 209).

Nevertheless, the standard deviations do increase with the means as in the data of Example 6, so let us take logs. We have:

	Normal	HCl	Lactic acid
Geometric mean	52.2	75.0	72.5
Multiplicative factor for standard deviation	1.21	1.28	1.19
	Pooled estimate 1.23		
Percent of Normal		144	139

Analysis of variance gives $F=6.90$ on 2 and 17 degrees of freedom, $P=0.0064$, so the conclusions are unchanged. It is perhaps a little more readily understandable to think in terms of percentages, which is what we are implicitly doing when using logarithms. The 'multiplicative factor for standard deviation' means that instead of quoting (for the HCl group) that the mean is 76.8 *plus or minus* 17.5 (SD) we can quote the geometric mean as 75.0 *multiplied or divided by* 1.23 (SD). The factor is found from the exponential function of the standard deviation of the natural logarithms of the data.

The non-parametric approach to the data (Siegel [3], p. 184) gives:

	Normal	HCl	Lactic acid
Median	53.5	78.0	69.0
Semi-interquartile range	6.3	8.5	12.5

Kruskall–Wallis statistic $=9.31$, which as χ^2 with 2 degrees of freedom gives $P=0.0095$ (Siegel [3], p. 184). The conclusion is unchanged, though the summary statistics (particularly the interquartile ranges) are unsatisfactory with the small samples (Armitage and Berry, p. 34).

Logarithms seldom do any harm, they are sometimes necessary for the correct analysis of data, and they can lead to easier interpretation of results.

Example 11

Each of six patients was dialysed using, in a random order, four different membranes. Complement activity, measured as factor B by radioimmunoassay in U/mol, was measured after 1 h.

Subject	Membrane			
	A	B	C	D
1	16.7	15.8	13.1	13.7
2	14.2	12.7	9.7	14.0
3	13.2	13.3	10.3	12.2
4	14.1	14.4	14.6	13.3
5	14.5	11.9	11.7	8.9
6	16.0	17.0	15.1	11.1

We shall ignore any possible effect of treatment order, though it could be important even after randomisation. As before let us summarise the data.

	Membrane			
	A	B	C	D
Mean	14.78	14.18	12.42	12.20
S.D.	1.31	1.94	2.23	1.94

Analysis of variance.

Source of variation	Sum of squares	DF	Mean square	F	*P*
Between membranes	29.6046	3	9.8682	4.28	0.023
Between subjects	36.3521	5	7.2704	3.15	0.036
Residual	34.5929	15	2.3062		
Total	100.5946				

The F-statistic between subjects is of no intrinsic interest; only the membranes concern us, and they are significantly different. Clearly membranes A and B give higher values than membranes C and D.

Notice, however, that the differences between subjects are important to the analysis. If they are ignored, and a one-way analysis of variance carried out, we get $F = 2.78$ on 3 and 20 degrees of freedom, $P = 0.068$, and miss the significant result. It is important to take into account all relevant features of the design. Rather more subjects would have been required in this study had it not been possible to give each subject all of the treatments.

Similarly, if a non-parametric approach is used, Kruskall–Wallis gives 6.29, which as a χ^2 with 3 degrees of freedom is non-significant. The correct test is the Friedman test (Siegel, p. 166) which gives $\chi^2 = 8.20$ with 3 degrees of freedom, and does indicate a significant difference between membranes.

Example 12

Creatinine clearance (in ml/min) was measured in 20 patients who had undergone 2 years treatment by continuous ambulatory peritoneal dialysis. These patients had suffered up to three episodes of peritonitis during that time.

Patient	1	2	3	4	5	6	7	8	9	10
Episodes of peritonitis	0	3	1	0	1	0	2	2	0	0
Creatinine clearance	18.3	15.2	25.8	19.9	19.5	18.3	13.0	22.3	16.2	22.3
Patient	11	12	13	14	15	16	17	18	19	20
Episodes of peritonitis	2	1	1	3	0	3	1	0	1	2
Creatinine clearance	13.4	13.1	25.5	22.0	20.2	13.3	19.9	21.4	23.4	19.8

To look for an association between the two variables we must notice that the explanatory variable (episodes of peritonitis) is categorical but ordered. An analysis of variance which ignores the ordering will be less powerful than one which takes it into account. Furthermore, by taking it into account we may estimate by how much the creatinine clearance increases or decreases per episode of peritonitis. We are therefore not only testing a null hypothesis: we are modelling the data by an equation. The total sum of squares is broken down into three components: that due to a linear relationship of creatinine clearance on number of episodes of peritonitis, that due to departures from linearity, and that due to variability within the groups with the same number of episodes of peritonitis (Armitage and Berry, p. 266). However, before constructing the analysis of variance table let us look as the individual groups.

Number of episodes of peritonitis	Number in group	Group mean	Group SD
0	7	19.51	2.08
1	6	21.20	4.79
2	4	17.12	4.65
3	3	16.83	4.57

There is no clear pattern, though there may be a tendency for the creatinine clearance to fall with increasing frequency of peritonitis.

Analysis of variance.

Source of variation	Sum of squares	DF	Mean square	F	*P*
Linear relationship	26.6621	1	26.6621	1.73	0.21
Non-linearity	31.9832	2	15.9916	1.04	0.38
Residual (within groups)	247.2227	16	15.4514		
Total	305.8680				

There is no significant relationship between the variables: the equation linking them (creatinine clearance = 20.4 − 1.1 for each episode of peritonitis) is such that the coefficient 1.1 is not significantly different from zero. Nevertheless, in many cases the difference between obtaining a significant result or not would depend on taking into account the ordering of the groups, which the one-way analysis of variance as applied to Example 10 does not do.

Final remarks

This article has not gone into some of the complications of the tests described. In particular it has assumed, as is often the case, that the reason for a significant result from the analysis of variance is apparent from looking at the means in the light of detailed knowledge of the treatments under investigation. If this is not so, there are several possible approaches, described by Armitage and Berry (p. 200). It cannot be emphasised too strongly, however, that multiple t-tests must not be used, *at least* until the analysis of variance has been performed and has yielded a significant result.

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Nephrol Dial Transplant (1988) 3: 478–481
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Data Analysis in Nephrology

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Analysis of Data in Nephrology IV. Relationships Between Continuous Variables

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Abstract. In this article the subjects of linear regression, non-linear curve fitting, and correlation are covered. Examples, as in the other articles in the series, are all taken from clinical or laboratory investigations in nephrology.

Key words: Continuous variables; Nephrology; Statistics

Introduction

There is no doubt what the first stage should be in looking for a relationship between a continuous response variable and a continuous explanatory variable. A graph should be drawn with the response variable on the y-axis and the explanatory variable on the x-axis. If the graph indicates that there is a straight line relationship between the variables, linear regression may be used to quantify the relationship. If there is evidence of curvature it may be possible to find a transformation, of one or both variables, which linearises the relationship. Otherwise a non-linear curve may be fitted to the data, but there should be a tenable *a priori* reason for choosing a particular curve.

Example 13

The results of measuring sodium clearance and renal plasma flow on 20 subjects were as follows:

Subject	Sodium clearance (ml/min)	Renal plasma flow (l/min)
1	0.40	0.58
2	0.34	0.77
3	0.40	1.25
4	0.57	1.14
5	0.39	1.02
6	0.32	1.00
7	0.52	1.03
8	0.34	0.60
9	0.28	0.87
10	0.33	1.03
11	0.39	0.88
12	0.44	1.07
13	0.47	1.41
14	0.25	0.73
15	0.34	0.67
16	0.51	0.95
17	0.46	1.27
18	0.42	1.10
19	0.44	0.97
20	0.49	1.37

It seems appropriate to take sodium clearance as the response variable (y) and renal plasma flow as the explanatory variable (x). The data are shown plotted in Fig. 1. There is no evidence of non-linearity, of any outliers, or of the scatter of the y-values changing as its value changes. Simple linear regression may therefore be performed (Armitage and Berry [1], p. 143), leading to the relationship

$$\text{sodium clearance} = 0.206 + 0.202 \times \text{renal plasma flow}.$$

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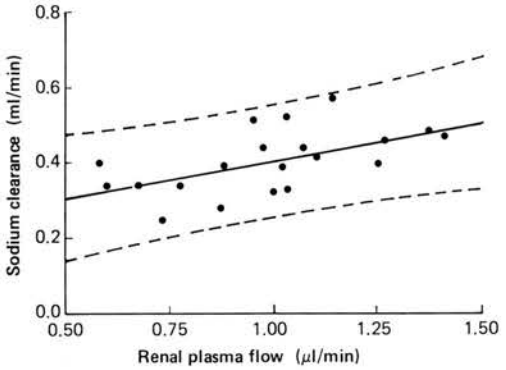

Fig. 1. Linear regression of sodium clearance on renal plasma flow for 20 subjects, with 95% prediction limits (Example 13).

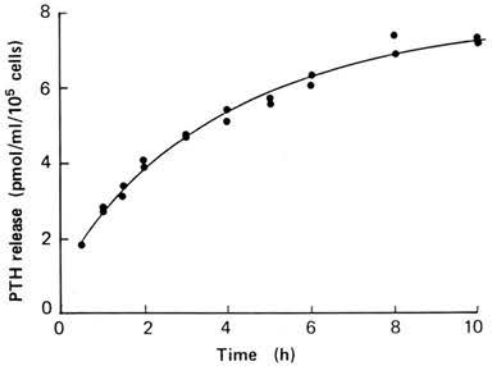

Fig. 2. Gompertz curve fitted to the time course of parathyroid hormone release (Example 14).

The fitted line is shown in Fig. 1. The estimate of the slope (0.202) has a standard error of 0.068, and a t-test therefore shows that there is a significant relationship between the variables. However, as with previous sets of data which we have described, it is profitable to do more than just carry out a significance test. For example, confidence limits for the slope can be obtained, or limits calculated, within which 95% of all sodium clearances will lie, given the renal plasma flow. These are also shown in Fig. 1. Thus if the renal plasma flow is 0.75 l/min sodium clearance values between about 0.2 and 0.5 ml/min would be expected, whereas for renal plasma flow of 1.25 l/m sodium clearance values lie between about 0.3 and 0.6 ml/min.

Example 14

Parathyroid hormone (PTH) release in single-cell suspensions was measured over time. Duplicate measurements were made with the following results:

Time (h)	PTH release (pmol/(10^5cells/ml))	
	Replicate 1	Replicate 2
0.5	1.81	1.85
1.0	2.79	2.70
1.5	3.07	3.35
2.0	3.86	4.08
3.0	4.71	4.67
4.0	5.38	5.08
5.0	5.66	5.49
6.0	6.00	6.25
8.0	6.81	7.35
10.0	7.28	7.14

Fig. 2 shows the graph of the data. Clearly there is a relationship, but equally clearly it is not a linear one. Can we find a transformation of the variables which linearises it? Access to computer graphics is clearly an advantage here, and it is easy with this facility to ascertain that no simple transformation (e.g. logarithms or reciprocals) will lead to a straight line. We must therefore decide on an appropriate curve. Since one with two parameters will not suffice, we must look among the large number of possible three-parameter curves. The data look as if they are approaching an asymptotic value as time increases, and a popular curve to fit in this instance is the Gompertz curve which can be written as

$$y = a - b\,e^{-cx}$$

where y will be the mean PTH release of the two replicates at each time x. There is not a great choice of easily available curve-fitting programs, but one is included in the BMDP package [2]. The program is shown in Fig. 3.

From the output we find that the fitted equation is

$$\text{PTH release} = 7.862 - 6.621\,e^{-0.2385 \times \text{time}},$$

and this curve (which is clearly a good fit to the data) is shown in Fig. 2. What can we gain from knowing this? Very little indeed. We do have an estimate of the final value for PTH release (7.862), and indeed the curve-fitting method also gives a standard error for this estimate (0.336), but to extrapolate beyond the range of the data in this way is very unwise. A curve with quite a different equation, which might fit the data equally well, could give quite a different answer.

There really has to be a purpose before a curve is fitted to a set of data: if there is an underlying biological model, as in pharmacokinetics, and a parameter of the curve is a well defined entity in the model, then it may be worth trying to estimate it, but curve-fitting just to see a curve passing through the points is no more useful an exercise than drawing a curve by hand, something no objective scientist would do. If the area under a curve is required, it is usually just as good to estimate it from the raw data as from a fitted curve. Nevertheless, to some data the Gompertz curve often fits well, and a comparison of estimated parameters between treatment groups or diagnostic

```
/PROBLEM   TITLE IS 'CURVE FITTING'.
/INPUT     VARIABLES ARE 3.
           FORMAT IS '(F3.1,2F4.2)'.
/VARIABLE  ADD=1.
           NAMES ARE TIME, REP1, REP2, MEAN.
/TRANSFORM MEAN=(REP1+REP2)/2.0.
/REGRESS   DEPENDENT IS MEAN.
           INDEPENDENT IS TIME.
           PARAMETERS ARE 3.
/PARAMETER INITIAL ARE 8.0,6.0,0.2.
/END
  5 181 185
 10 279 270
 15 307 335
 20 386 408
 30 471 467
 40 538 508
 50 566 549
 60 600 625
 80 681 735
100 728 714
```

BMDP program

```
SUBROUTINE P3RFUN(F,DF,P,X,N,KASE,NVAR,NPAR,IPASS,XLOSS,IDEP)
IMPLICIT REAL*8 (A-H,O-Z)
DIMENSION DF(NPAR),P(NPAR),X(NVAR)
DF(1)=1.
DF(2)=-DEXP(-P(3)*X(1))
DF(3)=-P(2)*X(1)*DF(2)
F=P(1)+P(2)*DF(2)
RETURN
END
```

Associated FORTRAN program

Fig. 3. BMDP and FORTRAN programs for the curve-fitting of Example 4.

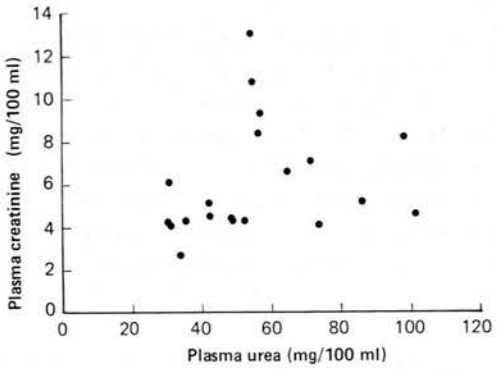

Fig. 4. Scatter diagram of plasma urea and plasma creatinine in 20 dialysis patients (Example 5).

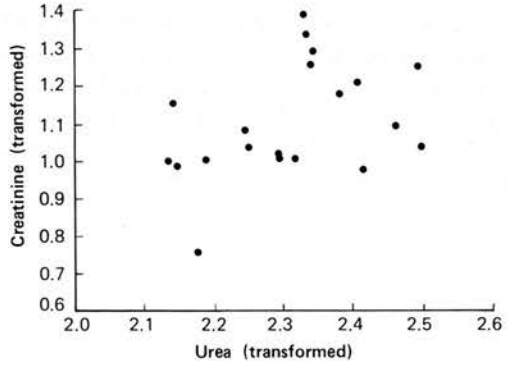

Fig. 5. Data of Fig. 4 with each variable transformed to give zero skewness.

groups may be useful, even though the individual estimates themselves should be treated with caution. It is for this reason, as well as to warn against it, that an example of curve-fitting has been included in this series.

Example 15

Twenty patients undergoing regular haemodialysis at home had their plasma urea and creatinine measured post-dialysis on at least 10 occasions, and the results averaged. Both are given below in mg/100 ml.

Here we are looking for a relationship between two continuous variables, but these variables are on an equal footing: they cannot reasonably be thought of as response and explanatory variables. Hence, regression analysis is not appropriate. But we start in the same way, by plotting the data: Fig. 4 shows a scatter diagram, and we have arbitrarily put urea on the x-axis and creatinine on the y-axis. The data do not appear to be particularly well represented by a linear relationship, and while a correlation coefficient could be calculated ($r = 0.23$) this is not a useful statistic. Perhaps if the data were transformed in some way, the relationship would be more nearly linear, and Fig. 5 shows the result of transforming the data so that the distributions are not skew: power transformations have been used, with urea (x) transformed to $(1-x^{-0.30})/0.30$ and creatinine (y) to $(1-y^{-0.54})/0.54$. It might be more appropriate to calculate the correlation coefficient now ($r = 0.40$), but such transformations are clumsy. It is better to relax the assumptions about the relationship between the variables and use a non-parametric measure of association.

Patient	1	2	3	4	5	6	7	8	9	10
Plasma urea	48.7	48.3	34.1	86.5	31.5	41.9	57.0	35.3	55.1	52.2
Plasma creatinine	4.27	4.42	2.63	5.19	4.05	5.07	9.23	4.22	10.74	4.25

Patient	11	12	13	14	15	16	17	18	19	20
Plasma urea	65.1	42.3	71.1	98.3	30.2	56.5	73.8	101.0	54.7	30.9
Plasma creatinine	6.56	4.56	7.07	8.15	4.23	8.40	3.99	4.57	12.94	6.06

Spearman's rank correlation coefficient (Siegel [3], p. 202) is 0.45, which is on the border of significance at the 5% level, a state of affairs not reached by the ordinary correlation coefficient. The rank correlation coefficient only assumes a relationship between the variables such that as one changes the other changes in a consistent direction. It can be used when there is a response variable and an explanatory variable, but of course the possibility of expressing one variable in terms of the other no longer exists.

The term 'averaged' which was used in describing the data has been glossed over. The arithmetic mean should not have been used as the data are not symmetrically distributed: the geometric mean, or the median since non-parametric methods are appropriate, would be more satisfactory. Notice, however, that the distribution of an observation within a subject might be quite different from that between subjects. Here we have only examined the relationship between urea and creatinine *between subjects:* we can say nothing on the basis of the data presented about whether the variables are related *within subjects.*

Final remarks

It is possible to be dogmatic about one thing when examining the relationship between two continuous variables: the data must be plotted first. After that it may be appropriate to fit a straight line, perhaps after transforming the data, or (less often) a curve, or to calculate a correlation coefficient. It is nearly always difficult to gain much information from a correlation coefficient, except that there is (or is not) a significant relationship between the variables.

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Nephrol Dial Transplant (1988) 3: 694–696
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Data Analysis in Nephrology

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Analysis of Data in Nephrology V. Contingency Tables

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Abstract. The first article in this series dealt with 2×2 contingency tables. We now return to contingency tables and discuss those formed by a dichotomous response variable and a discrete, but not dichotomous, explanatory variable. The logit transform is introduced.

Key words: Contingency tables; Logit; Nephrology; Statistics

Introduction

When a relationship between two dichotomous variables is to be investigated, the data take the form of a 2×2 contingency table which is easily analysed, although as shown in the first article of this series it is not always obvious immediately which of the several possible analyses is most appropriate. When the response variable is dichotomous but there are more than two categories for the explanatory variable, say k categories, we obtain a $2 \times k$ contingency table. The data may be presented in slightly different ways depending on the exact nature of the categories; specifically one may or may not decide to use the idea of relative risk, but the main thing to look for is whether or not the categories of the explanatory variable have an inherent ordering.

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Example 16

In a retrospective study of 100 patients with glomerulonephritis data were available on mode of presentation and whether or not the kidneys were functional after 2 years. Results were as follows:

Presentation	Number	Number on own renal function	Proportion on own renal function
Acute renal failure (ARF)	32	5	0.16
Asymptomatic proteinuria (AP)	30	17	0.57
Nephrotic syndrome (NS)	27	16	0.59
Recurrent haematuria (RH)	11	10	0.91

First a word about the way the data are tabulated. It is helpful to give the proportions which are being compared, but it is coincidental that the presentation categories have been ordered so that these proportions are in ascending order. In fact, since there is no inherent ordering in the categories they have been arranged in descending frequency of occurrence in the sample.

To carry out a significance test we arrange the data in the form of a 2×4 contingency table:

		ARF	AP	NS	RH	Total
Own renal function at 2 years	Yes	5	17	16	10	48
	No	27	13	11	1	52
	Total	32	30	27	11	100

The χ^2 value is 23.83 with 3 degrees of freedom: $P<0.001$ (Armitage and Berry [1], p. 205). In other words the four survival rates are significantly different. Difficulties may arise when the numbers in a category are too small for the χ^2 test to be appropriate. It may then be necessary to amalgamate categories in the explanatory variable, or to exclude a category from the analysis. If there is a category which obviously fulfils the function of a 'control', the proportion of positive responders in a small group may be compared to that in the control group via Fisher's exact test, but since the point of treating the table as a whole is to assign a single value to the probability of obtaining data as discrepant from the null hypothesis as was actually obtained, this should not be done lightly. In particular, if a non-significant χ^2 value is obtained for the table as a whole, even if expected values for the cell frequencies are small, no further tests should be carried out on individual 2×2 tables.

Example 17

In a study on the prognostic value of histology in glomerulonephritis 92 patients had their glomerular damage assessed as 'none', 'mild', 'moderate' or 'severe'. It was subsequently determined whether each patient was surviving on his own renal function 2 years later. Results were:

		Glomerular damage				
		None	Mild	Moderate	Severe	Total
Own renal function after 2 years	No	3	4	17	23	44
	Yes	16	14	12	2	47
	Total	19	18	29	25	92
Proportion dead or lost renal function at 2 years		0.16	0.22	0.59	0.92	
Approximate risk relative to group 'None'			1.5	7.6	61.3	

Again it is appropriate to give the proportions which are being compared, but because the response variable is an outcome and the explanatory variable a categorisation of a risk factor, and there is a category which acts in some ways as a reference point, it is appropriate to give also the odds-ratio for each category relative to that category.

The method which would most often be applied to such data would be to test the proportions for a linear trend as the categories become more severe. This certainly takes into account the ordered nature of the categories, and provides an adequate test in many circumstances. We shall perform this analysis, but then show a method which generalises better to more complex situations.

The χ^2 value for the table, ignoring ordering, is 32.89 with 3 degrees of freedom. This may be broken down (in an analogous way to the total sum of squares in an analysis of variance) into a χ^2 value of 31.08 with 1 degree of freedom and a χ^2 value of 1.81 with 2 degrees of freedom (Armitage and Berry, p. 372). The χ^2 with 1 degree of freedom represents the portion of the overall χ^2 value which can be attributed to a linear trend in proportions if the categories are regarded as equally far apart. The remaining χ^2 is a residual testing whether the linear trend is a sufficiently good model, so one hopes that it is small.

Experience has shown that in many situations it is not the proportions themselves which change linearly with category, but a function of the proportions: the logit. If p is a proportion, then logit $p = \log\{p/(1-p)\}$. Conversely, if $z = \text{logit } p$, $p = e^z/(1+e^z)$. When $p = 0.5$ $z = 0$ and when p tends to 0 or 1 z tends to minus or plus infinity. We have, in fact, met logit p before. For the 2×2 table

		Risk factor		
		Present	Absent	Total
Outcome	Unfavourable	a	b	n_3
	Favourable	c	d	n_4
	Total	n_1	n_2	N

We defined the log-odds-ratio $r = \log(ad/bc)$. But this is $\log(a/c) - \log(b/d)$, which in turn is $\log\{(a/n_1)/(1-a/n_1)\} - \log\{(b/n_2)/(1-b/n_2)\}$, which is logit p_1 − logit p_2 where p_1 is the proportion of those with the risk factor who have an unfavourable outcome, and p_2 is the proportion of those without the risk factor who have an unfavourable outcome. For the 2×2 table we merely tested whether r was zero, i.e. whether logit p was constant: for the $2 \times k$ table with k ordered categories we test to see whether a suitable description of the data is given by logit $p = a + bx$ where x is an index number assigned to the categories. For the table in the example we get logit $p = 2.28 - 1.40 \times$ severity, where none = 0, mild = 1, moderate = 2 and severe = 3. χ^2 values are given by the analysis (2) and these are used in exactly the same way as before: $\chi_1^2 = 35.08$ corresponds to the regression line and $\chi_2^2 = 2.06$ is the residual.

There is seldom enough precision in the data to decide conclusively which is the better method of analysis, but that using logits has two advantages: we end up with a

descriptive equation relating the response variable to the explanatory variable, and the method is one of a family which generalises to more complex situations.

Final remarks

It is to be expected that some of the ideas in this article have been less familiar than those in the previous ones in this series, particularly the discussion of the logit. Nevertheless, the basic ideas are worth thinking about, so that the power of this method of looking at data can be appreciated. The final article will show a further use of the logit, because it is now widely accepted as the most appropriate method of analysis when the outcome variable is dichotomous, and many statistical packages are now making it easy to use.

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LINGS ,A44216NDT 24.11.87 K00 [01] L02

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Nephrol Dial Transplant (1988) 0: 00–00
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Analysis of Data in Nephrology. VI The Generalised Linear Model

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Abstract. This is the last of six articles describing how to choose the appropriate statistical test to look for relationships between two variables. It shows that when the response variable is dichotomous and the explanatory variable is continuous it may not be appropriate to carry out a t-test. Some comments are made on extending the tests considered to more complex situations.

Key words: Logit; Nephrology; Nuisance variable; Statistics

Introduction

In the previous article in this series the logit of a proportion was introduced, and it was shown how this quantity could be used to test for a relationship between a dichotomous response variable, and a categorical explanatory variable. It is a simple matter to extend this idea to a continuous explanatory variable. The only reason that this is seldom done is the grip which the t-test holds in the medical literature.

Example 18

Fifteen mice were subjected to an experimental model of glomerulonephritis. Seven died within a week; the others survived for much longer periods. Their weights prior to the start of treatment were (in g):

Deaths	16.6	18.8	20.9	21.2	23.5	25.0	31.2	
Survivors	19.3	23.5	24.5	25.7	25.7	26.3	30.5	31.3

Few people would think twice: they would perform a t-test to compare the mean weights in the two groups. Some might prefer a non-parametric approach, but either way they would have neglected an important aspect of the data. Survival is clearly the response variable and weight is the explanatory variable, and while a t-test indeed performs a test of association between the variables it is not the most appropriate one; we can use a test which relates the response variable to the explanatory variable and not the other way round. If we let p be the probability that an animal will die (in 7 days) and we let x be its weight (on day zero) then we can see whether the data are consistent with the equation

$$\log\{p/(1-p)\} = a + bx$$

In other words we treat the logit of p almost as if it were a continuous variable y in linear regression. 'Almost as if' because the method of fitting the equation to the data is different. The easiest way to fit the data is through the program GLIM [1]. The program and its output are shown in Fig. 1. The fitted equation is logit p = 4.864 − 0.2071 × weight: the standard error for the slope is 0.1449, from which a t-test can be derived giving $t = 0.2071/0.1449 = 1.43$. An alternative is to use the difference in 'deviances' ($20.73 - 18.26 = 2.47$) which may be treated as a χ^2 deviate with 1 degree of freedom. The logic of this test is the same as that of linear regression or testing for a trend in proportions in relation to an ordered categorical variable: a model is first fitted which represents the null hypothesis, and we see whether the linear model gives a

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```
$UNITS 15   $DATA WEIGHT   $READ
16.6 18.8 20.9 21.2 23.5 25.0 31.2
19.3 23.5 24.5 25.7 25.7 26.3 30.5 31.3
$CALCULATE MOUSE=%GL(15,1) : DIED=%LE(MOUSE,7) : N=1
$ERROR BINARY N   $YVARIATE DIED
$FIT : WEIGHT  $DISPLAY E
        SCALED
 CYCLE  DEVIANCE      DF
    4    20.73        14

        SCALED
 CYCLE  DEVIANCE      DF
    4    18.26        13

      ESTIMATE      S.E.      PARAMETER
   1   4.864        3.520     %GM
   2  -0.2071       0.1449    WEIG
  SCALE PARAMETER TAKEN AS     1.000

$STOP
```

Fig. 1. GLIM program for the analysis of Example 18.

significantly better fit to the data. The two tests may give slightly different conclusions, and the second is usually to be preferred.

The advantage of fitting a model in this way, rather than merely testing hypotheses, is that we can use the fitted equation to make statements such as 'the probability that a mouse weighing 20 g will die under these experimental conditions is 0.33'. This sort of statement is most useful when the result of the analysis is a good fit, a significant result, and narrow confidence limits for any estimated parameter.

Final remarks

The test described in this article will not be in the repertoire of many nephrologists. It is hoped, however, that in essence it has been understood and accepted as the correct test in the context of the scheme of choosing tests promulgated in this series.

There are, unfortunately, many situations which have not been covered. When the relationship between more than two variables is to be investigated—and nearly always it should be—the choice of the correct analytical method is not so easy. The interpretation of the results of the analysis may be very complex. One common problem is how to extend one of the tests we have discussed when during a course of treatment the response variable is measured repeatedly. In effect this is introducing a second explanatory variable, time, and there are many other ways in which the numbers of explanatory variables may proliferate. Some of the methods covered, such as linear regression and its analogue using logits, generalise: the latter becoming logistic discriminant analysis. There are problems, however, when the explanatory variables are (as usually they are) intercorrelated. The analysis of variance can be formally extended, but both the calculations and the interpretation become much harder if sizes of groups differ. If the explanatory variables are all imposed by experimental design this is no problem, but if (as is often the case) the categorical variables which define the groups are measured as part of the study, difficulties arise.

One final example should suffice to show that it is very easy to come to the wrong conclusion by ignoring a possible explanatory variable. Let us return to the 2×2 table.

Example 19

One hundred patients with renal disease and one hundred controls were categorised as smokers or non-smokers with the following results:

	Smokers	Non-smokers
Renal disease	64	36
Control	42	58

A χ^2 test gives a highly significant result ($\chi^2 = 9.71$ with 1 degree of freedom): it is quite wrong because a third variable, sex, has been omitted from consideration. The full data might be as follows:

	Males		Females	
	Smokers	Non-smokers	Smokers	Non-smokers
Renal disease	60	20	4	16
Control	30	10	12	48

It is now obvious that for neither males nor females is there any evidence of a relationship between renal disease and smoking. The apparent relationship found in the original table arises because more males smoke than

females, and more males have renal disease than females. This difficulty can arise whether the variables are discrete or continuous, and the obverse can also happen: a true relationship can be missed because of interaction with a variable whose effect has been ignored.

Summary of the series of articles

(i) There are few set rules, but if you can look at the data graphically then do so.
(ii) Table 1 might be helpful.
(iii) Consider estimation rather than significance testing.
(iv) Don't be afraid of logarithms or logits.
(v) Be afraid of the effects of other variables.

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.A44216NDT 00 06888 0226 00235

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Journal of the neurological Sciences, 18 (1973) 111–129

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Data on the Distribution of Fibre Types in Thirty-six Human Muscles
An Autopsy Study

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(Received 3 August, 1972)

INTRODUCTION

It is a well-established fact that mammalian skeletal muscle fibres can be classified into fibre types on the basis of their metabolic and electrophysiological characteristics. The application of histochemical techniques provides a convenient method of studying the distribution of these fibre types in tissue sections. The use of one technique in particular, namely the myofibrillar ATPase reaction, has proved to be of value since it most directly reflects the physiological difference between fast and slow twitch fibres.

In this study, a large number of human muscles have been examined with regard to the respective distribution of fibre types within them, with three main objectives in view. Firstly, we wished to obtain reliable data on the fibre type constitution of normal muscles, so that valid comparisons may be made with clinical material in which it is thought that there may be a significant alteration in fibre type proportions. The difficulties inherent in obtaining adequate normal material by means of biopsy are well known. As a result, recourse has often been made to material from subjects who could not necessarily be regarded as unequivocally normal clinically, otherwise biopsy would not, as a rule, have been performed. Moreover, the data thus acquired are limited almost exclusively to the findings in muscles which are commonly biopsied. Our second objective was to find out whether there was any correlation between fibre type constitution on the one hand and the well-recognised clinical phenomenon of selective sparing or affection of particular muscles in the various forms of muscular dystrophy on the other. In this aspect of the work it was necessary to study many more muscles than could be obtained by means of biopsy procedures. Thus, both from the point of view of the extent of the survey and because it was important to adopt the strictest possible criteria of normality, autopsy material provided the only alternative.

Our interest lay not only in the numerical distribution of muscle fibres of the two main histochemical types in the various muscles studied, but also in their spatial distribution. It has been shown that the fibres of the normal motor unit occupy a territory which overlaps with the territories of other motor units of different physiological types, giving rise to the characteristic mosaic pattern of normal muscle (Edstrom and Kugelberg 1968). In diseases of muscle of neurogenic origin, the successive processes of denervation and reinnervation may modify this "chequerboard" pattern and give rise to groups of fibres of uniform type (Engel 1970). In assessing the significance of such fibre type grouping in biopsy material, it is important to have available data on the extent to which such grouping may appear normally in the context of differing fibre type proportions. The compiling of such data comprises the third object of this study.

MATERIALS AND METHODS

Samples of muscle were obtained at autopsy from 6 male subjects aged between 17 and 30 years. These subjects had all died suddenly from head injury (3), asphyxia (1), acute ventricular failure (1) and severe internal abdominal haemorrhage (1). The survey was limited to subjects of one sex, within a restricted age range, in order to obtain a group of comparable individuals. Table 1 shows the ages, weights and heights of the 6 subjects.

TABLE 1

THE AGES, WEIGHTS AND HEIGHTS OF THE SIX MALE AUTOPSY SUBJECTS

P.M. number	*Age (years)*	*Weight (kg)*	*Height (cm)*
1	28	98	183
2	18	78	198
3	20	78	183
4	17	74	183
5	30	82	188
6	18	61	183

Muscle specimens were removed from one side of the body, within 24 hr of death. In some cases, both superficial and deep parts of a particular muscle were sampled (see Table 2), and in several instances specimens were taken from more than one head within a single muscle. Blocks measuring approximately 1 cm^3 were frozen in dichlorodifluoro-methane (Arcton 12) cooled to $-150°$ C in liquid nitrogen. Cryostat sections were cut at a thickness of 10 μm and were stained to demonstrate the activity of myofibrillar ATPase at pH 9.5 (Hayashi and Freiman 1966). Sections were also stained with haematoxylin and eosin for histological examination.

Assessment of the muscle samples as regards fibre type proportions was made using the myofibrillar ATPase preparations. In each sample, three areas were selected at random and a total of 200 fibres were counted and classified as Type I or Type II. In this study, we did not differentiate between Type IIa and IIb (Brooke and Kaiser 1970) but classified them together as one type. The diameters of the same 200 fibres

TABLE 2

THE PERCENTAGES OF TYPE I FIBRES IN SAMPLES OF 36 HUMAN MUSCLES OBTAINED FROM 6 MALE ADULTS AT AUTOPSY

	Post-mortem No.					
	1	*2*	*3*	*4*	*5*	*6*
Abductor digiti minimi	N.T.	58.0	36.5	56.0	69.5	39.0
Abductor pollicis brevis	70.3	55.5	49.0	78.0	61.0	64.0
Adductor magnus (surface)	73.6	42.9	50.0	43.7	53.2	57.5
Adductor magnus (deep)	78.5	45.3	52.4	68.3	64.0	71.5
Adductor pollicis	86.0	69.5	72.0	92.5	83.0	79.1
Biceps brachii (surface)	53.3	34.4	34.4	49.0	45.0	37.4
Biceps brachii (deep)	61.0	50.5	37.4	57.9	56.0	40.5
Biceps femoris	80.5	73.0	54.3	73.4	56.7	63.6
Brachioradialis	59.8	32.7	27.8	43.5	45.2	29.5
Deltoid (superficial)	67.6	62.1	47.9	49.8	43.1	49.1
Deltoid (deep)	N.T.	64.5	43.9	76.8	58.0	61.8
1st Dorsal interosseus	N.T.	64.6	50.0	59.5	61.5	51.5
Erector spinae (surface)	100.0	57.9	26.7	62.7	50.5	52.6
Erector spinae (deep)	88.6	41.9	34.0	74.6	50.5	39.5
Extensor digitorum	49.0	42.2	48.4	45.5	56.2	42.5
Extensor digitorum brevis	74.5	46.0	32.5	37.5	45.0	36.5
Flexor digitorum brevis	N.T.	41.5	40.0	65.0	39.3	36.5
Flexor digitorum profundus	70.0	44.3	23.8	72.0	37.9	35.9
Frontalis	N.T.	N.T.	51.0	75.0	87.5	43.0
Gastrocnemius (lat. head surface)	46.0	45.5	35.2	46.0	37.7	50.7
Gastrocnemius (lat. head deep)	60.0	44.1	43.3	46.7	53.5	54.0
Gastrocnemius (medial head)	N.T.	47.6	49.3	53.4	46.9	56.9
Gluteus maximus	71.5	41.2	44.3	68.4	44.1	45.0
Iliopsoas	55.0	37.0	40.7	47.0	60.9	54.5
Infraspinatus	55.5	43.1	34.6	54.0	39.0	45.8
Latissimus dorsi	65.5	55.9	43.5	60.0	35.5	42.5
Orbicularis oculi	N.T.	21.0	14.0	23.5	18.0	0.5
Pectoralis major (clavic head)	57.3	39.3	31.9	46.5	45.5	33.0
Pectoralis major (sternal head)	60.7	29.2	27.4	57.5	44.6	39.5
Peroneus longus	76.4	57.5	49.0	63.0	69.8	59.5
Rectus abdominis	56.2	46.6	31.6	51.0	36.0	55.5
Rectus femoris (lat. head surface)	28.2	31.4	21.5	38.6	35.7	21.5
Rectus femoris (lat. head deep)	44.3	40.2	34.1	52.4	42.5	38.6
Rectus femoris (medial head)	N.T.	33.2	44.3	48.1	38.5	50.0
Rhomboid	61.9	38.5	43.3	47.5	45.0	31.2
Sartorius	59.5	48.3	38.5	51.9	60.5	39.0
Soleus (surface)	100.0	69.8	86.2	83.0	81.0	98.5
Soleus (deep)	100.0	87.5	90.6	77.0	83.0	96.0
Sternomastoid	46.5	37.0	30.1	25.8	32.4	39.2
Supraspinatus	72.0	46.0	41.4	85.0	64.5	46.9
Temporalis	N.T.	49.7	59.5	51.0	30.5	41.5
Tibialis anterior (surface)	80.5	79.0	57.6	70.0	85.6	67.6
Tibialis anterior (deep)	76.4	73.5	68.3	76.0	77.5	64.5
Trapezius	77.5	54.0	27.4	75.5	38.6	49.3
Triceps (surface)	58.2	31.0	20.0	37.5	34.0	14.5
Triceps (deep)	47.5	32.5	30.7	46.5	22.5	16.5
Vastus lateralis (surface)	45.5	33.8	41.4	49.8	23.3	32.9
Vastus lateralis (deep)	46.5	49.0	42.9	63.4	39.5	40.0
Vastus medialis (surface)	47.8	47.0	38.3	32.0	49.0	48.3
Vastus medialis (deep)	79.0	57.0	56.1	66.0	57.5	53.5

N.T. = Not taken.

in each sample were also measured; the data on fibre measurements forms the subject of a subsequent paper (Polgar, Johnson, Weightman and Appleton 1972).

In order to assess the amount of fibre type grouping in the samples, we adopted the method of Jennekens, Tomlinson and Walton (1971) who originated the idea of counting "enclosed" fibres in muscle specimens. "Enclosed" fibres are defined as those fibres which are completely surrounded by fibres of their own histochemical type. Since the packing of muscle fibres in fasciculi approximates to a hexagonal lattice array, it follows that on average 7 fibres of one type are required in order to give one "enclosed" fibre (see Fig. 1). If more fibres of the same type are added to such a group, it is clear that proportionally more fibres will be "enclosed" relative to the total number in the group. It is also clear that the number of "enclosed" fibres of any one type will tend to rise as the proportion of that fibre type in the sample rises. However, it has been shown recently, in rabbit and guinea pig muscle, that the fibres of some motor units are not randomly dispersed (James 1971). It was held to be possible, therefore, that the number of "enclosed" fibres might not be solely dependent upon the numerical proportion of that fibre type in the sample. Instead, it could be suggested that the spatial organisation of motor units might differ in two muscles which had the same fibre type proportions so that there was more uniform fibre type grouping and hence more "enclosed" fibres in one than in the other. In order to determine statistically whether there was any evidence of this sort of difference in organisation in the large number of muscles studied, a hypothetical model was devised for purposes of comparison. In the model situation, it was assumed that the arrangement of individual fibres conformed to a hexagonal lattice and that the spatial distribution of the fibres of the two histochemical types was random. On this basis, the expected number of "enclosed" fibres was calculated for a complete range of fibre type proportions. These data formed the basis for the assessment of any significant differences in the actual samples studied.

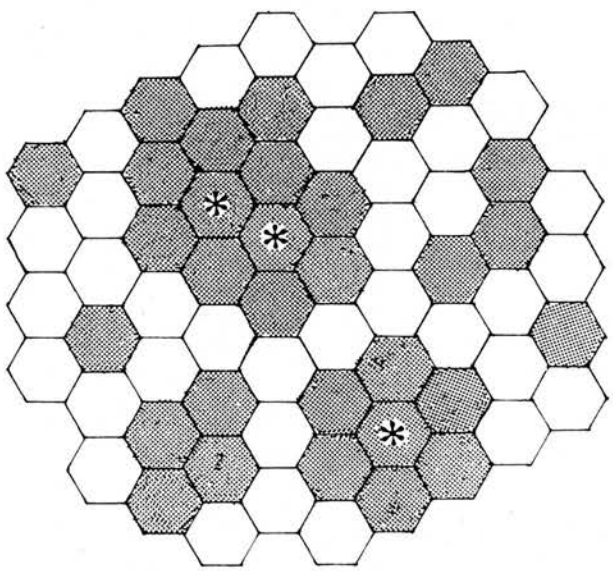

Fig. 1. A schematic representation of skeletal muscle as a hexagonal lattice showing two fibre types. "Enclosed" fibres (*) are those which are completely surrounded by fibres of their own fibre type.

TABLE 3

VARIABILITY OF TYPE I FIBRE PROPORTIONS BETWEEN THE SIX AUTOPSY SAMPLES OF INDIVIDUAL SKELETAL MUSCLES

Muscle	χ^2	*Degrees of freedom*	*Degree of significance*
Abductor digiti minimi	61.47	4	* * *
Abductor pollicis brevis	45.98	5	* * *
Adductor magnus (surface)	52.57	5	* * *
Adductor magnus (deep)	66.66	5	* * *
Adductor pollicis	47.57	5	* * *
Biceps brachii (surface)	27.87	5	* * *
Biceps brachii (deep)	38.57	5	* * *
Biceps femoris	50.54	5	* * *
Brachioradialis	66.33	5	* * *
Deltoid (surface)	37.48	5	* * *
Deltoid (deep)	48.79	4	* * *
1st Dorsal interosseus	13.58	4	*
Erector spinae (surface)	238.66	5	* * *
Erector spinae (deep)	197.74	5	* * *
Extensor digitorum	11.31	5	*
Extensor digitorum brevis	93.24	5	* * *
Flexor digitorum brevis	43.79	5	* * *
Flexor digitorum profundis	156.46	5	* * *
Frontalis	111.50	3	* * *
Gastrocnemius (lat. head surface)	14.64	5	*
Gastrocnemius (lat. head deep)	17.79	5	* *
Gastrocnemius (medial head)	5.97	4	Not sig.
Gluteus maximus	77.28	5	* * *
Iliopsoas	34.60	5	* * *
Infraspinatus	27.74	5	* * *
Latissimus dorsi	55.28	5	* * *
Orbicularis oculi	50.26	4	* * *
Pectoralis major (clavic head)	39.56	5	* * *
Pectoralis major (sternal head)	82.74	5	* * *
Peroneus longus	43.28	5	* * *
Rectus abdominis	44.55	5	* * *
Rectus femoris (lat. head surface)	25.51	5	* * *
Rectus femoris (lat. head deep)	16.25	5	* *
Rectus femoris (medial head)	16.42	4	* *
Rhomboid	47.54	5	* * *
Sartorius	38.04	5	* * *
Soleus (surface)	113.35	5	* * *
Soleus (deep)	72.73	5	* * *
Sternomastoid	24.73	5	* * *
Supraspinatus	125.92	5	* * *
Temporalis	38.65	4	* * *
Tibialis anterior (surface)	55.95	5	* * *
Tibialis anterior (deep)	13.81	5	*
Trapezius	161.65	5	* * *
Triceps (surface)	112.65	5	* * *
Triceps (deep)	70.93	5	* * *
Vastus lateralis (surface)	41.85	5	* * *
Vastus lateralis (deep)	32.42	5	* * *
Vastus medialis (surface)	20.11	5	* *
Vastus medialis (deep)	38.68	5	* * *

Significance levels for 5 degrees of freedom:

p	0.05	0.01	0.001
χ^2	11.070	15.086	20.517
	*	* *	* * *

TABLE 4

THE 95% CONFIDENCE LIMITS FOR MEAN FIBRE TYPE PERCENTAGE IN YOUNG MALE ADULT SUBJECTS

	Mean % type I fibres	*Mean % type II fibres*	*With 95% confidence the true mean % will lie between*			
			for type I fibres		*for type II fibres*	
Abductor digiti minimi	51.8	48.2	34.6	72.0	31.0	65.4
Abductor pollicis brevis	63.0	37.0	52.1	73.9	26.2	47.9
Adductor magnus (surface)	53.5	46.5	41.6	65.4	34.6	58.4
Adductor magnus (deep)	63.3	36.7	50.3	76.3	23.7	49.7
Adductor pollicis	80.4	19.6	71.3	89.5	10.6	28.7
Biceps brachii (surface)	42.3	57.7	33.9	50.7	49.3	66.2
Biceps brachii (deep)	50.5	49.5	40.5	60.5	39.3	59.6
Biceps femoris	66.9	33.1	56.0	77.8	22.2	44.0
Brachioradialis	39.8	60.2	30.0	52.6	47.4	73.0
Deltoid (superficial)	53.3	46.7	43.4	63.2	36.8	56.7
Deltoid (deep)	61.0	39.0	46.2	75.7	24.2	53.8
1st Dorsal interosseus	57.4	42.6	51.4	63.4	36.6	48.6
Erector spinae (surface)	58.4	41.6	33.3	83.5	16.5	66.7
Erector spinae (deep)	54.9	45.1	32.0	77.8	22.2	68.1
Extensor digitorum	47.3	52.7	41.8	52.8	47.2	58.2
Extensor digitorum brevis	45.3	54.7	29.3	61.3	38.7	70.6
Flexor digitorum brevis	44.5	55.5	33.6	55.4	44.6	66.4
Flexor digitorum profundis	47.3	52.7	26.9	67.8	32.2	73.2
Frontalis	64.1	35.9	31.2	97.0	3.0	68.8
Gastrocnemius (lat. head surface)	43.5	56.5	37.4	49.6	50.4	62.6
Gastrocnemius (lat. head deep)	50.3	49.7	43.3	57.2	42.8	56.7
Gastrocnemius (medial head)	50.8	49.2	45.6	56.0	44.0	54.4
Gluteus maximus	52.4	47.6	36.1	66.8	33.2	61.9
Iliopsoas	49.2	50.8	39.5	58.8	41.2	60.5
Infraspinatus	45.3	54.7	36.7	54.0	46.0	63.3
Latissimus dorsi	50.5	49.5	38.2	62.8	37.3	61.8
Orbicularis oculi	15.4	84.6	4.1	26.7	73.3	95.9
Pectoralis major (clavic. head)	42.3	57.7	32.2	52.3	47.7	67.8
Pectoralis major (sternal head)	43.1	56.9	28.5	57.8	42.2	71.5
Peroneus longus	62.5	37.5	52.5	72.6	27.4	47.5
Rectus abdominis	46.1	53.9	35.4	56.9	43.1	64.6
Rectus femoris (lat. head surface)	29.5	70.5	22.0	37.0	63.0	78.0
Rectus femoris (lat. head deep)	42.0	58.0	35.6	48.5	51.5	64.4
Rectus femoris (medial head)	42.8	57.2	34.1	51.5	48.5	65.9
Rhomboid	44.6	55.4	33.7	55.3	44.7	66.2
Sartorius	49.6	50.4	39.6	59.7	40.3	60.4
Soleus (surface)	86.4	13.6	74.5	98.4	1.6	25.5
Soleus (deep)	89.0	11.0	80.2	97.9	2.1	19.8
Sternomastoid	35.2	64.8	27.5	42.8	57.2	72.5
Supraspinatus	59.3	40.7	41.1	77.5	22.5	58.9
Temporalis	46.5	53.5	32.9	60.1	39.9	67.1
Tibialis anterior (surface)	73.4	26.6	62.6	84.1	15.9	37.4
Tibialis anterior (deep)	72.7	27.3	67.2	78.1	21.9	32.8
Trapezius	53.7	46.2	32.8	74.6	25.4	67.2
Triceps (surface)	32.5	67.5	16.5	48.6	51.4	83.5
Triceps (deep)	32.7	19.6	45.8	54.2	54.2	80.4
Vastus lateralis (surface)	37.8	67.3	19.6	45.8	52.1	72.3
Vastus lateralis (deep)	46.9	53.1	37.5	56.2	43.8	62.5
Vastus medialis (surface)	43.7	56.3	36.4	51.1	48.9	63.6
Vastus medialis (deep)	61.5	38.5	51.5	71.5	28.5	48.5

RESULTS

1. Fibre type constitution

The results shown in Table 2 list the percentages of Type I fibres in the 6 autopsy samples of each of the 36 muscles studied. Because more than one site was sampled in some cases, a total of 50 sample sites was arrived at, though in a few isolated instances, particular muscle samples were not obtainable. A chi-square test (Brandt–Snedecor) was used to compare the post-mortem samples in terms of fibre type proportion. The results of these tests are given in Table 3, together with the corresponding significance levels. It can readily be seen that in 41 out of the 50 sets of muscle sample there is a highly significant difference between samples taken from the different subjects. It is apparent from Table 2 that the difference cannot be attributed to the fact that any one subject is aberrant when compared with the others. Rather, the differences arise as a result of an inherently wide variation in the fibre type proportions

TABLE 5

COMPARISON OF FIBRE TYPE PROPORTIONS IN SUPERFICIAL AND DEEP AREAS OF CERTAIN SKELETAL MUSCLES

	No. of post-mortem subject					
	1	*2*	*3*	*4*	*5*	*6*
Adductor magnus	n.s.	n.s.	n.s.	*** S > D	* S > D	** S > D
Biceps brachii	n.s.	*** S > D	n.s.	n.s.	* S > D	n.s.
Deltoid	n.s.	n.s.	n.s.	*** S > D	** S > D	** S > D
Erector spinae	*** D > S	** D > S	n.s.	** S > D	n.s.	** D > S
Gastrocnemius (lat. head)	** S > D	n.s.	n.s.	n.s.	** S > D	n.s.
Rectus femoris (lat. head)	*** S > D	n.s.	** S > D	** S > D	n.s.	*** S > D
Soleus	n.s.	*** S > D	n.s.	n.s.	n.s.	n.s.
Tibialis anterior	n.s.	n.s.	* S > D	n.s.	* D > S	n.s.
Triceps	* D > S	n.s.	* S > D	n.s.	* D > S	n.s.
Vastus lateralis	n.s.	** S > D	n.s.	** S > D	*** S > D	n.s.
Vastus medialis	*** S > D	* S > D	*** S > D	*** S > D	n.s.	n.s.

S > D = Significantly more Type II fibres in surface area than in deep area of muscle.
D > S = Significantly more Type II fibres in deep area than in surface area of muscle.
n.s. = No significant difference between surface and deep areas.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

within the same muscle in a group of subjects whom we would expect to be comparable. In this small group it was impossible to draw any conclusions as to the influence of either age or physical build on the fibre type constitution of the skeletal muscles. Clearly, it would be of interest to investigate the possibility that fibre type proportions may vary with age or as a result of differences in occupation. It is also possible that there may be a significant difference in fibre type proportions between male and female subjects, since Brooke and Engel (1969) have shown a sex difference in the relative sizes of the two main fibre types. Until these questions have been answered, we would consider it safer to use data on mean fibre type proportions to compare only subjects matched for age and sex.

Table 4 sets out the approximate 95 % confidence limits for the mean fibre type percentages in the case of both Type I and Type II fibres. This treatment of the data provides a convenient basis of comparison of routine biopsy material. It can be seen that the variance in fibre type proportions of the individual muscles differs widely. Several reasons for this may be suggested. Firstly, in large muscles with complex physiological functions, the problem of sampling error will be greater than in smaller and less complex muscles. Also, in the case of muscles receiving their innervation from different segmental nerves, the chance of significantly different fibre type proportions occurring in different areas of the same muscle will be increased. In this study, every effort was made to minimise sampling error by taking muscle specimens from exactly the same site at each autopsy.

In several muscles, samples were taken from both superficial and deep sites, and in the case of gastrocnemius, pectoralis major and rectus femoris, samples were taken from both heads of the muscle. The specimens thus obtained have been compared in order to give some indication of the variation to be expected within individual muscles. The results of these comparisons are given in Tables 5 and 6. It can be seen that a significant difference in fibre type proportion between deep and superficial areas of the muscles thus sampled is not a constant finding, but where it does occur it is most frequently due to the fact that the superficial area contains a higher proportion of

TABLE 6

COMPARISON OF FIBRE TYPE PROPORTIONS IN DIFFERENT HEADS OF 3 SKELETAL MUSCLES

	No. of post-mortem subject					
	1	*2*	*3*	*4*	*5*	*6*
Gastrocnemius (lateral head *vs.* medial head)	n.s.	n.s.	* * L > M	n.s.	n.s.	n.s.
Pectoralis major (clavicular head *vs.* sternal head)	n.s.	* C > S	n.s.	* C > S	n.s.	n.s.
Rectus femoris (lateral head *vs.* medial head)	* * L > M	n.s.	* * * L > M	* L > M	n.s.	* * * L > M

All muscle samples in this table were taken from the surface of the muscle.
L > M = Significantly more Type II fibres in lateral head than in medial head.
C > S = Significantly more Type II fibres in clavicular head than in sternal head.
n.s. = not statistically significant.

Type II fibres (see Table 5). Similarly, there was no consistently significant difference in the fibre type proportions of samples taken from different heads of gastrocnemius, pectoralis major or rectus femoris (see Table 6).

2. Fibre type grouping

In the theoretical model of muscle fibre distribution, it was assumed that the fibres of each fibre type were randomly arranged. Thus the probability that a fibre at a given locus will be, say, a Type I fibre, can be expressed as a function of p, the proportion of Type I fibres in the sample. It follows, therefore, that the probability of a Type II fibre occurring at a given locus in the same sample will be 1-p. The probability of a Type I being "enclosed" by 6 other Type I fibres will be p^6, so that in a sample of N fibres the expected number of Type I "enclosed" fibres will be Np^7. For small values of p a Poisson approximation can be used to calculate the expected number of "enclosed" fibres and its standard deviation; for larger values of p the Normal approximation may be used. As the number of "enclosed" fibres is binomially distributed, the standard deviation is $\sqrt{Np^7(1-p^6)}$.

TABLE 7

THE RELATIONSHIP BETWEEN THE OBSERVED NUMBER OF MUSCLE FIBRES OF A PARTICULAR FIBRE TYPE AND THE EXPECTED NUMBER OF "ENCLOSED" FIBRES OF THE SAME TYPE

Number of fibre type observed	*Number of "enclosed" fibres of that type expected*	*99.9% ± Confidence limits*
0	0.0	0.0
20	0.0	0.0
40	0.0	0.3
60	0.0	0.7
80	0.3	2.0
100	1.6	4.0
120	5.6	7.6
140	16.5	12.5
160	41.9	18.4
170	64.1	20.7
180	95.7	22.1
190	139.7	20.1
195	167.5	16.1
200	200.0	0.0

Table 7 sets out the expected number of "enclosed" fibres for a complete range of fibre type proportions, taking $N=200$, the actual number of fibres examined. From this table it is apparent that comparatively small numbers of enclosed fibres of a given fibre type are to be expected until the percentage constitution of that fibre type rises above 70%. From this point onwards, there is a steep rise in the expected number of "enclosed" fibres until, of course, this reaches 100% in a muscle sample consisting solely of that particular fibre type.

The actual number of "enclosed" fibres observed in any one sample was compared with the number expected in a hypothetical sample of identical fibre type constitution

TABLE 8

COMPARISON OF OBSERVED NUMBER OF "ENCLOSED" FIBRES WITH THE EXPECTED NUMBER OF "ENCLOSED" FIBRES

P.M. number	*Type I*						*Type II*					
	1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*	*4*	*5*	*6*
Abductor digiti minimi	NT						NT					−
Abductor pollicis brevis	+ + +		+								+	
Adductor magnus (surface)								−				
Adductor magnus (deep)				− −	−	−						
Adductor pollicis			− −									
Biceps brachii (surface)								− −	− −			−
Biceps brachii (deep)				−								
Biceps femoris	+				−	−						
Brachioradialis								−	−			
Deltoid (surface)		−										
Deltoid (deep)	NT					−	NT					
1st Dorsal interosseus	NT		+	−	+ + +		NT		+ +		+ +	
Erector spinae (surface)												
Erector spinae (deep)									− −			−
Extensor digitorum												−
Extensor digitorum brevis	+ + +		+ +		+ + +	+	+ + +	+ + +		+ +	+ + +	+ + +
Flexor digitorum brevis	NT						NT					
Flexor digitorum profundis			+ +	+					+			
Frontalis	NT	NT		+ + +	− − −		NT	NT				
Gastrocnemius lat. head (surface)												
Gastrocnemius lat. head (deep)						+ +	+ +					
Gastrocnemius medial head	NT						NT					
Gluteus maximus					+	−						
Iliopsoas								− −				
Infraspinatus							+ + +			− −		
Latissimus dorsi												
Orbicularis oculi	NT						NT	− −	− − −	+ +	− − −	
Pectoralis major clavicular head								− −		+		−
Pectoralis major sternal head								−				
Peroneus longus					+							

(*continued*)

TABLE 8 (*contd.*)

P.M. number	*Type I*						*Type II*					
	1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*	*4*	*5*	*6*
Rectus abdominis												
Rectus femoris lat. head (surface)												− −
Rectus femoris lat. head (deep)										−		
Rectus femoris medial head	NT						NT					
Rhomboid	−											− − −
Sartorius	+ + +											−
Soleus (surface)		+	− −			− − −						
Soleus (deep)				+	+							
Sternomastoid			+						− −			
Supraspinatus				+					−			
Temporalis											− −	
Tibialis anterior (surface)			+	−	− −							
Tibialis anterior (deep)							+ +	− −				
Trapezius									− −		− −	
Triceps (surface)	−											−
Triceps (deep)											+	
Vastus lateralis (surface)												
Vastus lateralis (deep)											−	− −
Vastus medialis (surface)										− − −	+ +	
Vastus medialis (deep)					−							

− = observed number enclosed fibres lower than expected $p < 0.05$.
− − = observed number enclosed fibres lower than expected $p < 0.01$.
− − − = observed number enclosed fibres lower than expected $p < 0.001$.
+ = observed number enclosed fibres higher than expected $p < 0.05$.
+ + = observed number enclosed fibres higher than expected $p < 0.01$.
+ + + = observed number enclosed fibres higher than expected $p < 0.001$.
NT = sample not taken.

as the sample under scrutiny. Figs. 2 and 3 express graphically the results of the comparison of observed and expected "enclosed" fibre numbers which are set out in detail in Table 8. In those muscle samples where significantly high numbers of "enclosed" fibres occur, it can be concluded that the degree of grouping of fibres of uniform fibre type is greater than would occur in a randomly distributed population. One muscle in particular stands out in this respect, namely, extensor digitorum brevis. This muscle contained a significant amount of uniform fibre type grouping in samples from all the subjects ($p < 0.001$ in 4 out of the 6 subjects). In some cases, both fibre types showed evidence of grouping; in others, only one fibre type was involved. Abductor pollicis

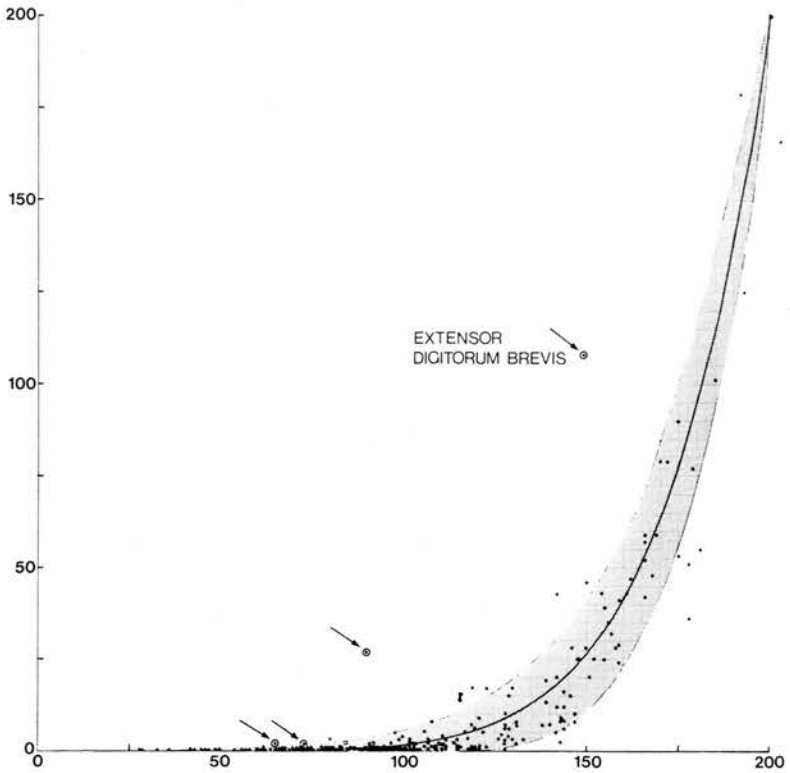

Fig. 2. The relationship between the number of enclosed Type I fibres and the total number of Type I fibres observed. The shaded area shows the formal 99.9% limits about the mean expected enclosed fibres. The only muscle which is above the limit more than once is extensor digitorum brevis which appears 4 times out of 6 post-mortem specimens.

brevis, 1st dorsal interosseus and flexor digitorum profundus showed less consistent evidence of fibre type grouping. In the other muscles, it was either extremely sporadic or non-existent. There was no suggestion that any individual subject showed significantly more instances of uniform fibre grouping than any other.

In those muscle samples where significantly low numbers of "enclosed" fibres were recorded, it would seem reasonable to conclude that the degree of dispersion of fibres of that fibre type is greater than would occur if the population were randomly distributed. However, the method employed is less suitable for detecting evidence of this than for detecting fibre type grouping. In the vast majority of cases it was found that there was no significant difference between the observed and the expected number of "enclosed" fibres, which would strongly suggest that in most normal muscles the two main fibre types are in fact randomly dispersed.

3. Fibre type constitution of muscle selectively affected in the muscular dystrophies

One of the objectives of this study was to examine the possibility that the extent to which individual muscles are affected in dystrophic processes might in some way be influenced by their fibre type composition. For example, there is evidence that myo-

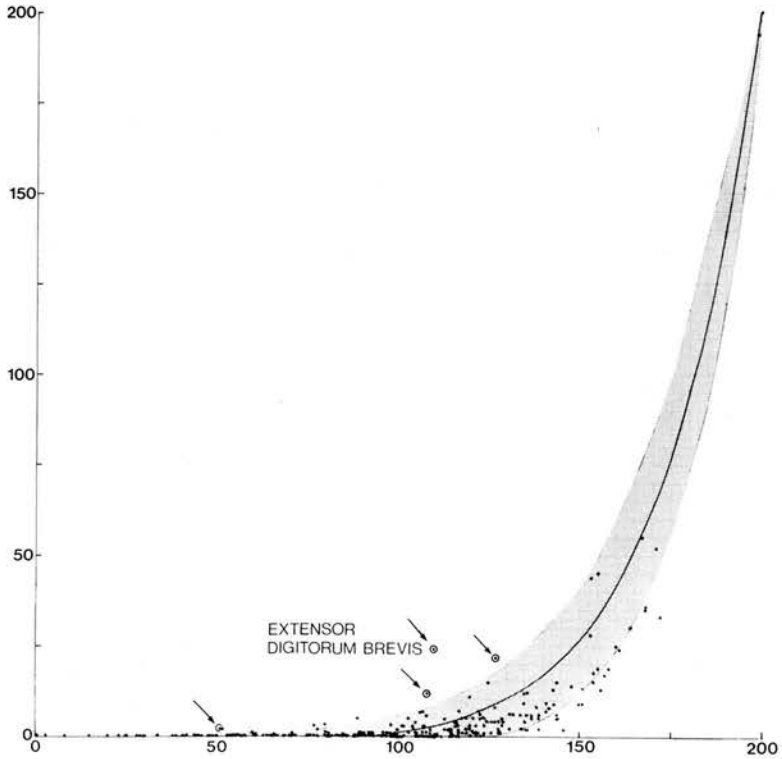

Fig. 3. The relationship between the number of enclosed Type II fibres and the total number of Type II fibres observed. The shaded area shows the formal 99.9 % limits about the mean expected enclosed fibres. The only muscle which is above the limit more than once is extensor digitorum brevis which appears 4 times out of 6 post-mortem specimens.

tonic dystrophy involves a selective atrophy of Type I fibres (Engel 1970), and for this reason it could be argued that muscles with a high proportion of Type I fibres might be most severely affected. In Duchenne dystrophy, however, there is no such evidence of the particular involvement of one fibre type, yet the phenomenon of selective sparing of muscles is just as apparent as in myotonic dystrophy.

Table 9 lists those muscles which are usually selectively affected or spared in Duchenne dystrophy and myotonic dystrophy, together with their respective percentage constitutions of Type I fibres as measured in this study. In general, the order in which the muscles are listed reflects the order in which they are affected in the disease, though naturally there is some variation between individual cases.

From this Table it is immediately obvious that there is no simple correlation between the fibre type composition of individual skeletal muscles and their being affected or spared in either Duchenne dystrophy or myotonic dystrophy. There is some suggestion that the overall tendency is for muscles with high Type II fibre percentages to be more severely affected in Duchenne dystrophy, but tibialis anterior, for instance, with its very low percentage of Type II fibres, is an obvious exception to this. In spite of its apparent predilection for Type I fibres, there was no evidence to show that myotonic dystrophy selectively affects muscles with a high proportion of that fibre type.

TABLE 9

THE RESPECTIVE PERCENTAGES OF TYPE I FIBRES FOUND IN THOSE MUSCLES SELECTIVELY INVOLVED EARLY (ACCORDING TO CLINICAL EXAMINATION) IN DUCHENNE DYSTROPHY AND MYOTONIC DYSTROPHY, AND IN THOSE RELATIVELY SPARED

Affected early	*Type I* %	*Relatively spared*	*Type I* %
Duchenne dystrophy			
(Upper limb)		(Upper limb)	
Pectoralis major	42.3	Deltoid–surface	53.3
Biceps brachii–surface	42.3	–deep	60.9
–deep	50.5	Infraspinatus	45.3
Brachioradialis	39.8	Adductor digiti minimi	51.8
Triceps–surface	32.5	Adductor pollicis brevis	62.9
–deep	32.7	1st Dorsal interosseus	57.4
(Lower limb)		(Lower limb)	
Vastus medialis–surface	43.7	Gastrocnemius–lat. head surface	43.5
–deep	61.5	–lat. head surface	50.3
Vastus lateralis –surface	37.8	–medial head	50.8
–deep	46.9	Soleus–surface	86.4
Tibialis anterior	73.4	–deep	89.0
Peroneus longus	62.5		
Adductor magnus–surface	53.5		
–deep	63.3		
Myotonic dystrophy			
Temporalis	46.5	Extensor digitorum brevis	45.3
Sternomastoid	35.2	Flexor digitorum brevis	44.5
Flexor digitorum profundus	47.3	Adductor digiti minimi	51.8
Extensor digitorum	47.3	Adductor pollicis brevis	62.9
Tibialis anterior–surface	73.4	1st Dorsal interosseus	57.4
–deep	72.7		
Peroneus longus	62.5		
Gastrocnemius–lat. head surface	43.5		
–lat. head deep	50.3		
–medial head	50.8		

DISCUSSION

Although there have been several recent studies concerning the relative sizes of the two main fibre types in human skeletal muscle, comparatively little attention has been directed towards the assessment of the numerical ratios of these fibre types. Certainly, no previous study has had as its main objective a systematic analysis of this parameter in a wide range of human muscles. From the wealth of data thus accumulated, it was thought that some correlation between fibre type constitution and function might emerge, as has been the case in studies of the fast and slow twitch muscles of small mammals. However, from the outset one is only too well aware of the complexity of human musculature, and the fact that anatomical complexity is seldom fortuitous but develops in response to a functional demand. Thus in very few instances can one simple function be assigned to a particular muscle. Far more frequently, muscles fulfil

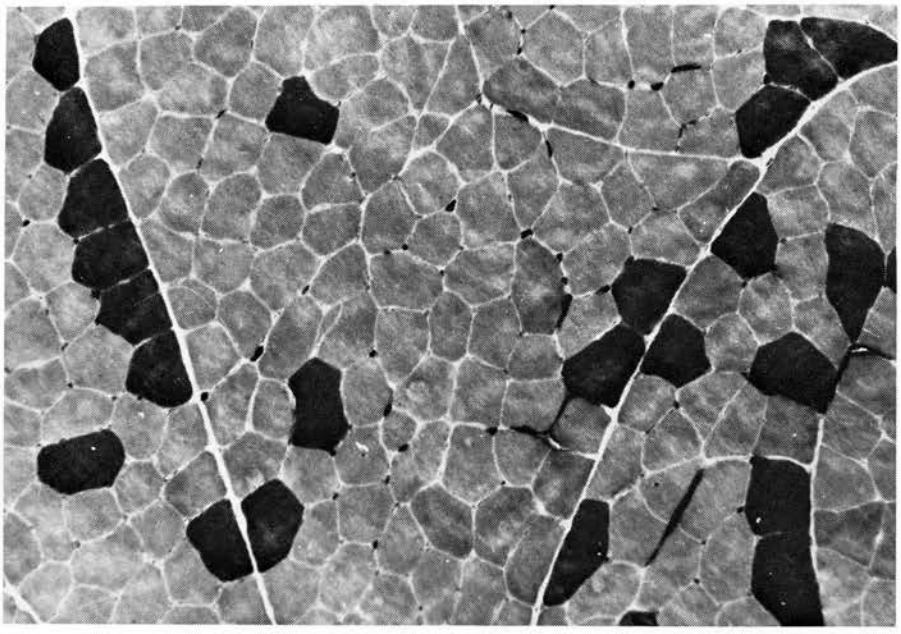

Fig. 4. Adductor pollicis from post-mortem No. 4 (myofibrillar ATPase preparation, × 150). Note the large number of enclosed Type I fibres, which is nevertheless within normal limits for a muscle with *c.* 80% Type I fibres.

both a postural function involving tonic activity and also participate in movements involving phasic activity.

However, it has been found that muscles with an almost exclusively postural function, such as soleus and, to a lesser extent, tibialis anterior, have particularly high proportions of Type I fibres (*c.* 87% and 73% respectively) which was in accordance with expectation. The similarly high percentage (*c.* 80%) of Type I fibres in adductor pollicis (see Fig. 4) may well be explained on the basis of its function of sustained contraction in the act of gripping.

At the other end of the scale, as far as fibre type constitution is concerned, are found the orbicularis oculi, sterno-mastoid, triceps and the lateral head of rectus femoris. Since the act of blinking is perhaps the best example of a phasic activity, the high proportion of Type II fibres in orbicularis oculi was to be expected. The function of the triceps in the predominantly phasic activity of extending the forearm is well enough established and the high proportion (67.5%) of Type II fibres was to be expected in this muscle also. Similarly, the sterno-mastoid, controlling the more delicate and precise of the movements of the head, has little postural function and its high Type II fibre constitution reflects this. More surprisingly, an even higher proportion (*c.* 70%) of Type II fibres was found in the superficial region of the lateral head of the rectus femoris (see Fig. 5). However, it is likely that the function of assisting the iliopsoas to support the pelvis on the femur is performed mainly by the medial sector of this muscle, and the lateral sector is primarily involved in the flexion of the femur and extension of the knee.

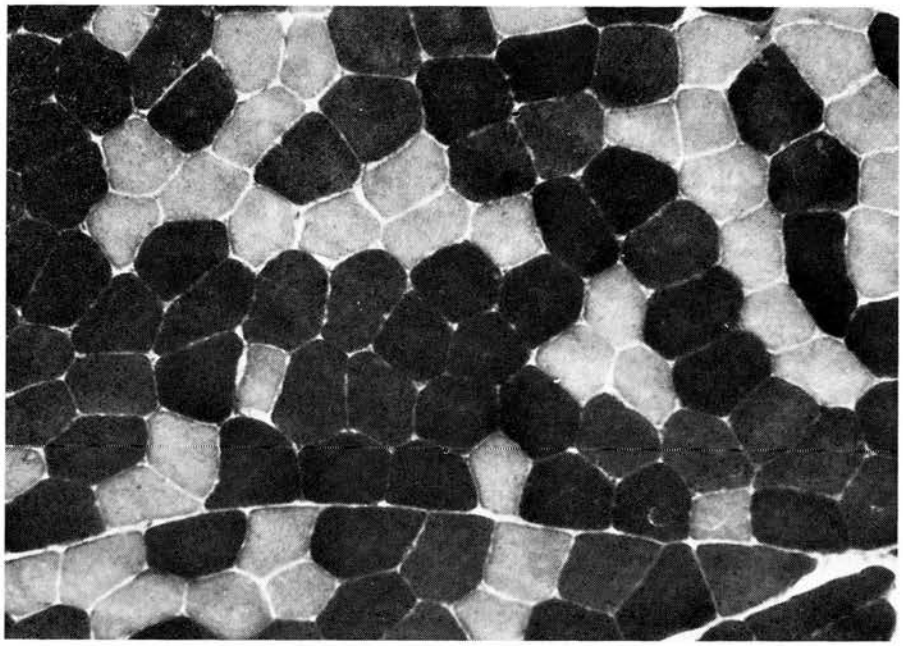

Fig. 5. Rectus femoris (lateral head) from post-mortem No. 2. (myofibrillar ATPase preparation, × 150). Note the close adherence to a hexagonal lattice array in some areas of the section. The mean Type II fibre percentage is *c.* 70% for this muscle.

The vast majority of the muscle samples had between 40% and 70% of Type I fibres, and in comparatively few muscles was there a striking predominance of either fibre type.

The large variation in fibre type percentages between different autopsy samples of individual muscles has already been discussed. Brooke and Engel (1969) found an even wider variation in their study of biceps brachii and vastus lateralis, perhaps because their series included a wider range of ages and some subjects who had previous histories of neuromuscular disease. Nevertheless, in the case of male subjects, the mean percentage of Type I fibres for both vastus lateralis and biceps brachii approximated to 37.5% (ratio of Type I : Type II fibres = 0.6). This agrees quite closely with our values of *c.* 38% for vastus lateralis and *c.* 42% for biceps brachii. The samples obtained by Brooke and Engel were all from biopsy material and hence have been assumed to be most directly comparable with our samples of the superficial areas of these muscles. In the case of the female subjects studied by Brooke and Engel, there was a definitely greater proportion of Type I fibres in biceps brachii as compared with the male subjects, whereas female vastus lateralis tended to have a smaller proportion of Type I fibres than in the male. The classification of their subjects into groups of similar clinical status rather than into similar age groups precluded any comment as to the possible influence of age on fibre type proportions, but re-examination of their data with this point in mind failed to detect any such influence in their series. However, it has been shown recently (Moore, Rebeiz and Holden 1971) that the average fibre diameter in a range of muscles underwent a gradual but steady increase until the late third or early

fourth decade, thereafter slowly diminishing in size through middle and old age. Until reliable data exist to prove or disprove such an effect of age on fibre type constitution, we consider it safer to use data on mean fibre type for age and sex.

In the part of the present study which was concerned with the spatial distribution of the two main fibre types, we hoped to gain greater insight into the organisation underlying the familiar "chequerboard" appearance of muscle sections. In order to do this, the actual muscle samples have been compared with a mathematical model to test the hypothesis that the distribution of the fibre types was random. In the great majority of cases this was in fact found to be so, since there was no significant difference in the number of "enclosed" fibres actually observed and the numbers predicted on the basis of the "hexagonal lattice" model.

There are many difficulties in formulating a mathematical model of a biological situation. In this particular instance it is evident that the model is not a strictly accurate one since the arrangement of the muscle fibres themselves inevitably sometimes departs from the hexagonal lattice of the model. Also, it was necessary to count fibres outside the region whose fibre type proportion was calculated, in order to determine whether a fibre on the edge of the region is enclosed. Thus the possibility arises that the fibres involved may have a slightly different fibre type proportion p from that noted which may materially alter the expected number of "enclosed" fibres. If these extra fibres have a proportion of only 0.1 different from the ones counted, the difference in expectation of the number of "enclosed" fibres is of the same order as the standard deviation. These inaccuracies make the technique slightly over-sensitive. However, it has the virtue of providing a simple and objective method of assessing whether the fibre type distribution in any muscle sample is random or not. In this respect, it has proved to have some predictive power, since it singled out extensor digitorum brevis as being the one instance of a muscle with a consistently non-random spatial distribution of fibre types.

In a study of this particular muscle in subjects whose ages ranged from 2 months to over 80 years, it has been shown that while a normal mosaic pattern apparently exists in early childhood, definite instances of uniform fibre type groupings have been recorded within the first decade of life (Jennekens, Tomlinson and Walton 1972). The occurrence in apparently normal older subjects of the characteristic features of denervation atrophy in this muscle has led to the adoption of the view that the muscle is particularly prone to recurrent cycles of denervation and re-innervation from a comparatively early stage of life. In the present study, unequivocal evidence of grouped denervation atrophy was noted in the 2 oldest subjects. Since groups of fibres of uniform type were seen in all the subjects, it is likely that the process of re-innervation can keep pace with that of denervation in younger persons. Isolated instances of grouped fibre atrophy were also noted in abductor pollicis brevis and 1st dorsal interosseus, two muscles in which there was also an excess of uniform fibre type grouping as reflected in the abnormally high number of "enclosed" fibres in some samples. The mathematical model thus seems to provide a means of quantitating the histological consequences of denervating processes.

In young adults, the spatial distribution of the different fibre types appears to be random in the great majority of muscles. The special case of extensor digitorum brevis and, to a lesser extent, abductor pollicis brevis, however, leads one to question whether

increasing age might not similarly affect other muscles. It might be expected that distal muscles would be particularly susceptible to sub-clinical neuropathic conditions of the "dying-back" variety. However, in this study it is of interest to note the lack of fibre type grouping in flexor digitorum brevis as compared with extensor digitorum brevis, and in adductor pollicis as compared with abductor pollicis brevis, which are pairs of muscles situated approximately equidistant from the segment of the spinal cord supplying their nerves.

The question of sub-clinical pressure or entrapment neuropathies operating in the same way to modify the spatial distribution of fibre types is a possibility in the case of abductor pollicis brevis since it is supplied by the terminal branch of the median nerve which is affected in the carpal tunnel syndrome. Compression of the ulnar nerve is also common, especially at the elbow, and this could affect the 1st dorsal interosseus muscle. Similarly, it was suggested by Jennekens *et al.* (1972) that the lateral terminal branch of the deep peroneal nerve supplying extensor digitorum brevis might be compressed intermittently at its entrance to the muscle by the pressure of ill-fitting shoes.

It is perhaps unwise to regard the occurrence of re-innervation and the occurrence of groups of fibres of uniform fibre type as inseparable factors. This study has provided evidence that a random mosaic pattern of fibre distribution can be considered to be the norm for a wide range of muscles. Even where uniform fibre type grouping occurs, it is likely that this is secondary to an arrangement of fibre types which was originally random. However, it is impossible to exclude the converse situation in which denervation and re-innervation take place but fail to produce groups of fibres of uniform type. For uniform fibre grouping to occur, at least two conditions must be satisfied. Firstly the denervated fibres must occupy a fairly compact territory, which pre-supposes the involvement of more than one motor unit, since the fibres of any one unit are scattered (Edstrom and Kugelberg 1968). Secondly, the process of re-innervation must be effected either by a single motor unit or by more than one unit of the same type. If the first condition does not obtain, the collateral re-innervation of disseminated atrophied fibres is unlikely to produce uniform fibre type grouping, though repetition of the same chain of events might eventually have this effect. If the second condition is not fulfilled and two motor units of different physiological type participate in the re-innervation process, it is likely that a mosaic pattern will result in much the same way as it does in the initial innervation process. Thus it is quite possible that the successive phases of denervation and re-innervation may be more common than is generally supposed since they will remain undetected unless the process gives rise to physiological or histological anomalies. Situations may also be envisaged in which the process of re-innervation leads to the fibre type constitution of a particular muscle becoming significantly different from normal, while at the same time the number of enclosed fibres is merely consistent with the new fibre type constitution, and would give no indication that re-innervation had taken place. The examination of biopsy material for evidence of previous denervation should therefore take into account not only the number of enclosed fibres observed, but also the proportion of the respective fibre types which the muscle examined contains.

ACKNOWLEDGEMENTS

We should like to thank Professor J. N. Walton for his helpful advice and criticism throughout the course of this study. We are also grateful to Mrs. Valerie Giles, Miss Jennifer Allison and Mrs. Doreen Hargreaves for invaluable technical assistance.

SUMMARY

Samples of skeletal muscle were taken from 50 sites in each of 6 previously normal male autopsy subjects aged between 17 and 30 years. The respective percentages of Type I and Type II fibres were calculated and showed that there was a wide variation in fibre type proportions between the 6 samples in almost all the muscles studied. Examination of the mean fibre type proportions of each muscle revealed that predominantly tonic muscles had a high percentage of Type I fibres and predominantly phasic muscles had a high percentage of Type II fibres. Most of the muscles studied were known to fulfil both tonic and phasic functions, however, and showed no striking preponderance of either fibre type.

The spatial distribution of the fibre types was examined in order to determine whether this was random or not. The number of "enclosed" fibres observed in the actual samples was compared statistically with the number expected to occur in a hexagonal lattice model, assuming a random distribution. In the great majority of muscles, the distribution of the fibre types was in fact random, though isolated instances of grouping of fibres of uniform type were noted in some distal muscles and more regularly in extensor digitorum brevis.

The methods used in the quantitative assessment of the proportions and spatial distribution of the respective fibre types in normal muscle have obvious applications in the study of neuromuscular disease.

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Journal of the neurological Sciences, 1973, 19: 307–318

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Data on Fibre Size in Thirty-six Human Muscles

An Autopsy Study

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(Received 5 January, 1973)

INTRODUCTION

The considerable number of studies of human muscle fibre size already undertaken by other workers have all had at least one objective in common. Namely, they have attempted to overcome the errors inherent in a subjective assessment of the phenomena of fibre atrophy and hypertrophy by accurate measurement. Previous studies, however, have been largely limited to the small number of muscles which are commonly biopsied, since the main purpose has been to compare normal fibre sizes with the corresponding measurements made on pathological material. The collection of normal data for the purposes of comparison with routine biopsy specimens has, in fact, been one of the reasons for this study but it is by no means its only objective.

We have been more particularly interested in being able to define the amount of variation to be expected within a group of normal subjects, comparable in terms of age and sex. Autopsy material has been used, since it was obviously impossible to obtain samples of the large number of muscles studied by any other means. Moreover, we were thus able to exclude subjects in which there was any real likelihood of neuromuscular disease, whereas biopsy material is generally only obtainable if there is in fact such a probability.

The relative sizes of the two main histochemical fibre types have been assessed in each muscle in order to see if there was any evidence of a correlation with the function of the various muscles in tonic and phasic activity. A previous study of fibre type proportions using identical material (Johnson, Polgar, Weightman and Appleton 1973) has shown that there is a relationship between the predominant function of a muscle and its fibre type constitution. It was therefore of interest to see if there was a similar correlation as far as the relative sizes of the two fibre types were concerned. We also wished to find out if there was a significant difference in the amount of variability in fibre size shown by different muscles and also by the two fibre types within any one muscle. It was thought that such differences might arise as a result of particular muscles having a greater capacity for hypertrophy in response to functional demands than others.

MATERIALS AND METHODS

Samples of muscle were obtained at autopsy from 6 male subjects aged between 17 and 30 years. All 6 subjects had died suddenly from head injury (3), asphyxia (1), acute ventricular failure (1) and severe internal abdominal haemorrhage (1). Table 1 shows the ages, weights and heights of the 6 subjects.

The samples of muscle were removed from one side of the body within 24 hr of death. In several instances, samples were taken from both superficial and deep parts of a muscle, and in some cases specimens were taken from more than one head of the same muscle. Blocks measuring approximately 1 cm^3 were frozen in dichlorodifluoromethane (Arcton 12) cooled to $-150°C$ in liquid nitrogen. Cryostat sections were cut at a thickness of 10 μm and were stained to demonstrate the activity of myofibrillar ATPase at pH 9.5 (Hayashi and Freiman 1966).

TABLE 1

AGES, WEIGHTS AND HEIGHTS OF THE 6 MALE AUTOPSY SUBJECTS

P.M. number	*Age (yrs)*	*Weight (kg)*	*Height (cm)*
1	28	98	183
2	18	78	198
3	20	78	183
4	17	74	183
5	30	82	188
6	18	61	183

In each muscle sample three areas were selected at random and were photographed at a constant magnification, so as to include a total of at least 200 fibres in the three photographs. The diameters of 200 or more fibres were measured using the method of Song, Shimada and Anderson (1963). These authors maintained that the most valid estimate of the true diameter of a muscle fibre is obtained by first defining the "major axis" or longest diameter, and then by measuring the "minor axis" which is the greatest diameter falling at right angles to the major axis. The minor axis has been shown to be the dimension least likely to be affected by distortion due to imperfect transverse sectioning or compression of the fibre itself.

The 200 fibres in each sample were classified as Type I or Type II, no distinction being made between Type IIa or IIb fibres in this study (Brooke and Kaiser 1969). The numerical proportions of the fibre types of the same 200 fibres were calculated and form the subject matter of a previous communication (Johnson *et al.* 1973).

STATISTICAL METHODS

The different numbers of Type I and Type II muscle fibres occurring in each post-mortem sample of a particular muscle necessitated the use of an analysis of variance for disproportionate sub-class numbers (Snedecor and Cochran 1967). This method gave the best estimate of the difference between the mean diameters of the muscle fibres. In 49 out of the 50 muscle sites an interaction effect between fibre type and

post-mortem was present, and in calculating the variance ratio the interaction variance was used.

To demonstrate the amount of variability in fibre size for each muscle site, 95% confidence limits were estimated for the mean fibre diameters based on the variance between post-mortems (Snedecor and Cochran 1967).

RESULTS

The mean fibre diameters of each fibre type in the 50 muscle sites studied are set out in alphabetical order in Table 2.

It has been shown in previous studies (Brooke and Engel 1969; Jennekens, Tomlinson and Walton 1971), that in the commonly biopsied muscles, Type II fibres are generally larger than Type I fibres in male subjects. The fact that this finding does not seem to apply to female subjects has been attributed to the contention that women do not generally carry out so much heavy physical work. In the present study, it was of interest to determine whether the previous observations on a small number of muscles in male subjects was equally applicable to the large number of sites sampled here. Type II fibres were in fact larger on average than Type I fibres in 44 out of the 50 muscle sites investigated.

In order to make simpler the appreciation of the differences between the mean diameters of the two fibre types, they have been listed in order of magnitude in Table 3. In only 6 muscle sites was the difference in favour of Type I fibres, and in only 1 of these cases was the difference statistically significant. In 15 cases out of 44 instances in which the Type II mean was greater than the Type I mean, the difference was significant. Muscles in which there was a non-significant difference between the means included vastus lateralis, deltoid, gastrocnemius and triceps. Of the commonly biopsied muscles, only biceps brachii showed a significant difference between fibre type means as estimated in this study.

In general, the muscles showing a significant difference constituted a very heterogeneous group. In an attempt to rationalise the occurrence of a significant difference, we wondered if it could be correlated with the function of the muscle concerned or with the numerical proportions of the fibre types within the muscle, two factors which have themselves shown some correlation. Table 4 lists those muscles in which a significant difference in mean diameters was found and also their proportion of Type I fibres. A few non-significant cases have also been included for purposes of comparison. From this table it is apparent that Type II fibres may be significantly larger than Type I fibres both in muscles with a high proportion of Type I fibres or of Type II fibres. Consequently, since muscles with a high proportion of Type I fibres are predominantly tonic, and those with a high proportion of Type II fibres are predominantly phasic, there is no evident correlation with mode of action. To illustrate this lack of correlation, the instances of muscles showing the largest Type II advantage in terms of fibre diameter include tibialis anterior (73% Type I fibres) and adductor pollicis (80% Type I) which are both mainly tonic, and brachioradialis (40% Type I) which is mainly phasic. Also, in contrast to tibialis anterior, the soleus

TABLE 2

MEAN FIBRE DIAMETERS (μm) OF TYPE I AND TYPE II FIBRES IN 50 MUSCLE SAMPLES FROM EACH OF 6 AUTOPSY SUBJECTS

Muscle	*Type I*	*Type II*
Abductor digiti minimi	38.1	44.6
Abductor pollicis brevis	41.5	53.4
Adductor magnus (surface)	65.0	65.7
Adductor magnus (deep)	61.7	62.0
Adductor pollicis	47.7	63.0
Biceps brachii (surface)	50.5	55.8
Biceps brachii (deep)	49.8	52.1
Biceps femoris	60.9	67.4
Brachioradialis	50.2	63.5
Deltoid (surface)	51.6	58.6
Deltoid (deep)	49.1	52.1
1st dorsal interosseus	50.0	67.0
Erector spinae (surface)	60.0	57.0
Erector spinae (deep)	61.6	53.6
Extensor digitorum	46.8	51.3
Extensor digitorum brevis	39.8	53.9
Flexor digitorum brevis	59.3	65.0
Flexor digitorum profundus	49.1	60.9
Frontalis	20.6	27.0
Gastrocnemius (lat. head surface)	48.3	50.5
Gastrocnemius (lat. head deep)	48.4	50.5
Gastrocnemius (medial head)	65.0	66.0
Gluteus maximus	56.0	61.6
Iliopsoas	54.5	51.7
Infraspinatus	44.6	47.7
Latissimus dorsi	51.4	61.7
Orbicularis oculi	21.7	24.2
Pectoralis major (clavic. head)	53.9	60.2
Pectoralis major (sternal head)	55.1	62.2
Peroneus longus	53.5	56.7
Rectus abdominis	43.0	55.5
Rectus femoris (lat. head surface)	64.7	73.6
Rectus femoris (lat. head deep)	68.7	74.0
Rectus femoris (medial head)	64.6	74.6
Rhomboid	56.7	55.6
Sartorius	47.6	49.0
Soleus (surface)	66.0	68.0
Soleus (deep)	69.5	70.6
Sternomastoid	50.2	53.3
Supraspinatus	52.1	53.7
Temporalis	37.4	25.6
Tibialis anterior (surface)	53.8	66.6
Tibialis anterior (deep)	56.0	64.6
Trapezius	59.3	66.1
Triceps (surface)	56.3	63.5
Triceps (deep)	57.2	62.8
Vastus lateralis (surface)	63.1	63.2
Vastus lateralis (deep)	64.6	63.7
Vastus medialis (surface)	61.6	64.7
Vastus medialis (deep)	62.0	65.7

TABLE 3

DIFFERENCES BETWEEN MEAN DIAMETERS (μm) OF TYPE I AND TYPE II FIBRES STARTING WITH HIGHEST TYPE I ADVANTAGE

	Type II minus Type I	*F*	*p*
Temporalis	−11.89	6.46	n.s.
Erector spinae (deep)	− 7.99	7.41	<0.05
Erector spinae (surface)	− 3.04	<1	n.s.
Iliopsoas	− 2.82	2.78	n.s.
Rhomboid	− 1.06	<1	n.s.
Vastus lateralis (deep)	− 0.84	<1	n.s.
Vastus lateralis (surface)	0.14	<1	n.s.
Adductor magnus (deep)	0.28	<1	n.s.
Adductor magnus (surface)	0.66	<1	n.s.
Gastrocnemius (medial head)	1.01	<1	n.s.
Soleus (deep)	1.07	<1	n.s.
Sartorius	1.41	1.18	n.s.
Supraspinatus	1.52	<1	n.s.
Soleus (surface)	2.05	<1	n.s.
Gastrocnemius (lat. head deep)	2.06	1.38	n.s.
Gastrocnemius (lat. head surface)	2.19	1.57	n.s.
Biceps brachii (deep)	2.39	2.34	n.s.
Orbicularis oculi	2.62	2.99	n.s.
Deltoid (deep)	3.02	<1	n.s.
Sternomastoid	3.07	1.09	n.s.
Vastus medialis (surface)	3.07	5.10	n.s.
Infraspinatus	3.13	3.05	n.s.
Peroneus longus	3.19	2.75	n.s.
Vastus medialis (deep)	3.70	2.41	n.s.
Extensor digitorum	4.57	4.91	n.s.
Biceps brachii (surface)	5.25	10.54	<0.05
Rectus femoris (lat. head deep)	5.30	1.57	n.s.
Triceps (deep)	5.52	3.42	n.s.
Gluteus maximus	5.62	4.59	n.s.
Flexor digitorum brevis	5.66	13.68	<0.05
Frontalis	6.39	12.07	<0.05
Pectoralis major (clavic. head)	6.42	3.17	n.s.
Abductor digiti minimi	6.49	108.60	<0.001
Biceps femoris	6.61	8.46	<0.05
Trapezius	6.79	3.74	n.s.
Deltoid (surface)	7.01	3.56	n.s.
Pectoralis major (sternal head)	7.07	5.32	n.s.
Triceps (surface)	7.12	6.03	n.s.
Tibialis anterior (deep)	8.68	5.51	n.s.
Rectus femoris (lat. head surface)	8.86	5.47	n.s.
Rectus femoris (med. head)	9.92	16.85	<0.01
Latissimus dorsi	10.27	11.09	<0.05
Flexor digitorum profundus	11.76	11.62	<0.05
Abductor pollicis brevis	11.96	35.44	<0.01
Rectus abdominis	12.46	15.56	<0.05
Tibialis anterior (surface)	12.74	12.51	<0.05
Brachioradialis	13.07	14.76	<0.05
Extensor digitorum brevis	14.13	106.67	<0.001
Adductor pollicis	15.26	17.59	<0.01
1st dorsal interosseus	17.02	101.79	<0.001

TABLE 4

COMPARATIVE FIBRE TYPE DIAMETERS VERSUS FIBRE TYPE PROPORTIONS

Muscle	*Type II minus Type I (μm)*	*p*	*Type I fibre (%)*
Temporalis	−11.89	n.s.	46.5
Erector spinae (deep)	− 7.99	<0.05	54.9
Vastus lateralis (surface)	0.14	n.s.	37.8
Soleus (surface)	2.05	n.s.	86.4
Biceps brachii (surface)	5.25	<0.05	42.2
Flexor digitorum brevis	5.66	<0.05	44.5
Frontalis	6.39	<0.05	64.1
Abductor digiti minimi	6.49	<0.001	51.8
Biceps femoris	6.61	<0.05	66.9
Rectus femoris (medial)	9.92	<0.01	42.8
Latissimus dorsi	10.27	<0.05	50.5
Flexor digitorum profundus	11.76	<0.05	47.3
Abductor pollicis brevis	11.96	<0.01	63.0
Rectus abdominis	12.46	<0.05	46.1
Tibialis anterior (surface)	12.74	<0.05	73.4
Brachioradialis	13.07	<0.05	39.8
Extensor digitorum brevis	14.13	<0.001	45.3
Adductor pollicis	15.26	<0.01	80.4
1st dorsal interosseus	17.02	<0.001	57.4

muscle, which is comparable on the grounds of similar tonic and postural function, showed a non-significant difference between the sizes of Type I and Type II fibres.

It may be mentioned here that all the small muscles of the hands and feet that were sampled in this study (abductor pollicis brevis, abductor digiti minimi, adductor pollicis, 1st dorsal interosseus, extensor digitorum brevis and flexor digitorum brevis) had significantly larger Type II fibres, irrespective of their widely differing fibre type constitutions and functions. The reason for this is not immediately obvious but would not seem to be purely coincidental. In a previous paper we have discussed the occurrence of severely atrophied fibres in abductor pollicis brevis and extensor digitorum brevis in normal subjects (Johnson *et al.* 1973). In those cases where grouped fibre atrophy was noted in this study, those areas were excluded from the samples of 200 fibres which were measured and were not therefore responsible for influencing the result.

Another possibility which we were interested in exploring was that Type II fibres in general might prove to show greater variability in diameter measurement than Type I fibres. It is known that Type II fibres participate mainly in phasic activity and therefore it might be expected that they would be the fibres which would increase in size in response to the demands placed upon them by exercise to a greater extent than the Type I fibres. In a random group of normal individuals such as the ones included in our study, chosen only because they fell within a particular age range, it is reasonable to assume that the amount and type of exercise taken by the individuals would vary considerably. Because of this, it was thought that those muscles most involved in strenuous activity might well show the greatest variation in fibre size, especially in their Type II fibres.

TABLE 5

95% CONFIDENCE LIMITS FOR THE MEAN DIAMETERS (μm) OF TYPE I AND TYPE II FIBRES IN A SINGLE SUBJECT, FOR EACH MUSCLE SITE

Muscle site	*Type I*		*Type II*	
	lower	*upper*	*lower*	*upper*
Abductor digiti minimi	26	52	28	60
Abductor pollicis brevis	14	70	27	79
Adductor magnus (surface)	50	80	48	83
Adductor magnus (deep)	45	78	34	90
Adductor pollicis	31	65	44	80
Biceps femoris	29	74	27	83
Biceps brachii (surface)	31	70	31	72
Biceps brachii (deep)	45	77	52	82
Brachioradialis	35	69	26	98
Deltoid (surface)	33	70	27	90
Deltoid (deep)	29	68	49	57
1st dorsal interosseus	35	66	47	86
Erector spinae (surface)	41	78	32	83
Erector spinae (deep)	49	73	34	74
Extensor digitorum	36	58	34	68
Extensor digitorum brevis	28	53	40	67
Flexor digitorum brevis	42	78	45	84
Flexor digitorum profundus	28	76	32	85
Frontalis	14	28	19	33
Gastrocnemius (lat. head surface)	43	54	40	61
Gastrocnemius (lat. head deep)	37	60	42	60
Gastrocnemius (medial head)	36	92	43	90
Gluteus maximus	31	84	32	87
Iliopsoas	39	69	34	70
Infraspinatus	30	60	24	70
Latissimus dorsi	31	74	30	92
Orbicularis oculi	11	31	14	35
Pectoralis major (clav. head)	35	75	25	94
Pectoralis major (sternal head)	29	86	25	96
Peroneus longus	40	67	42	71
Rectus abdominis	32	55	35	75
Rectus femoris (lat. head surface)	56	76	50	96
Rectus femoris (lat. head deep)	51	85	56	93
Rectus femoris (medial head)	48	80	55	94
Rhomboid	39	75	37	73
Sartorius	39	57	34	63
Soleus (surface)	47	87	45	95
Soleus (deep)	56	82	44	97
Sternomastoid	36	64	29	78
Supraspinatus	31	76	28	76
Temporalis	25	51	10	50
Tibialis anterior (surface)	38	71	46	85
Tibialis anterior (deep)	36	76	48	80
Trapezius	47	71	48	86
Triceps (surface)	42	77	29	95
Triceps (deep)	37	84	25	97
Vastus lateralis (surface)	40	87	48	78
Vastus lateralis (deep)	44	85	58	70
Vastus medialis (surface)	50	73	55	74
Vastus medialis (deep)	47	77	50	81

There was, however, no statistical evidence to show that the amount of variability of fibre diameters was significantly greater in Type II fibres than in Type I fibres. The normal ranges, which were calculated for both fibre types, are listed in Table 5. Some of the chief muscles involved in flexion and extension of the arms as in lifting, pulling and pushing, namely the biceps brachii, triceps and pectoralis major, showed wide variation in size. However, the gastrocnemius, which provides much of the propelling force in walking, running and leaping, showed a fairly narrow range of fibre size in the lateral head though not in the medial head.

Having seen that the relative sizes of Type I and Type II fibres cannot be correlated with the function of the muscles concerned, it still remained to be seen if there was any correlation of function or fibre type constitution with the absolute sizes of the muscle fibres. However, neither Type I nor Type II fibres showed any such correlation. Nevertheless, as might be expected from the large number of muscles in which there was no significant difference between the mean diameters of Type I and Type II fibres, muscles with small Type I fibres tended to have small Type II fibres, and large Type I fibres generally accompanied large Type II fibres (Fig. 1).

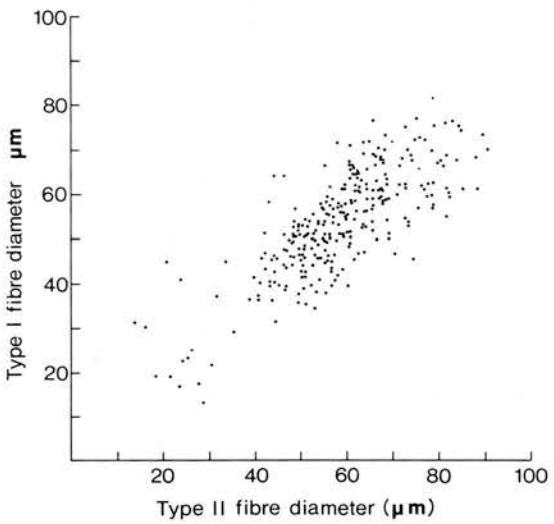

Fig. 1. Relationship of Type I and Type II fibre diameters in individual skeletal muscles from the 6 autopsy subjects.

As might also be expected, the larger muscles tended to have larger fibres. The facial muscles sampled had consistently small fibres of both types as did abductor digiti minimi. However, some of the other small muscles of the hands and feet had surprisingly large diameter fibres. For example, the Type I fibres of flexor digitorum brevis had a mean diameter of 59.4 μm as compared with a value of 61.6 μm for Type I fibres from the surface of vastus medialis. The Type II fibres of the 1st dorsal interosseus had a mean diameter of 67.0 μm while that of Type II fibres in biceps femoris was 67.4 μm. Conversely, some fairly large muscles had remarkably small mean fibre diameters, *e.g.* sartorius with a mean Type I diameter of 47.6 μm and a Type II mean of 49.0 μm. However, the muscles showing the largest mean fibre diameters for both

TABLE 6

THE DIFFERENCES IN THE MEAN FIBRE DIAMETERS (μm) OF TYPE I AND TYPE II FIBRES FROM DEEP AND SURFACE AREAS OF SEVERAL SKELETAL MUSCLES

Muscle	*Type I (Deep–Surface)* D_I–S_I	*Type II* D_{II}–S_{II}	$(D_I–S_I)$ $-(D_{II}–S_{II})$
Adductor magnus	−3.3	−3.7	0.4
Biceps brachii	−0.8	−3.6	2.9
Deltoid	−2.5	−6.5	4.0
Erector spinae	1.5	−3.5	5.0
Gastrocnemius	0.1	0.0	0.1
Rectus femoris	4.0	0.4	3.6
Soleus	3.6	2.6	1.0
Tibialis anterior	2.1	−2.0	4.1
Triceps	0.9	−0.7	1.6
Vastus lateralis	1.4	0.4	1.0
Vastus medialis	0.4	1.1	−0.7
Mean difference	+0.7	−1.4	+2.1
"t"$_{10}$ value	0.99	1.40	3.67
Level of significance	n.s.	n.s.	$p<0.01$

types were rectus femoris (medial and lateral heads), adductor magnus, tibialis anterior, soleus, biceps femoris, vastus lateralis, vastus medialis and the medial head of gastrocnemius. In all these muscles the mean fibre diameter for both types exceeded 60 μm.

Subsequently, we wished to find out if there was a significant difference in the mean fibre diameters of either Type I or Type II fibres in surface and deep areas in those muscles from which multiple samples were taken. Table 6 lists the differences in Type I diameters from deep and surface areas of these muscles (D_I–S_I) and also the differences between the mean diameters of the Type II fibres (D_{II}–S_{II}). The initial impression given by the table is that Type I fibres have a tendency to be larger in the deep area as compared with the surface area of a given muscle. However, a *t*-test revealed that this tendency was non-significant. Similarly, the apparent tendency of Type II fibres to be larger on the surface rather than in the deeper layers of the muscle, was found to be non-significant also. However, a significant difference ($p< 0.01$) was found in the *relative* sizes of the two fibre types in surface and deep areas. The difference in the mean diameter of the fibre types is greater in the deep areas than it is in the surface areas. This "difference between differences" can be expressed as (D_I–S_I)–(D_{II}–S_{II}) or as (D_I–D_{II})–(S_I–S_{II}), its algebraic equivalent.

Similar analyses carried out on the medial vs lateral head of the rectus femoris and the sternal vs clavicular head of pectoralis major revealed no significant differences. In these instances, the individual mean fibre diameters for each autopsy subject were used instead of the overall mean. The results of thus comparing the medial and lateral heads of gastrocnemius are given in Table 7. Here there is convincing evidence that the diameter of both fibre types is greater in the medial head of gastrocnemius than in its lateral head.

TABLE 7

COMPARISON OF MEAN FIBRE DIAMETERS (μm) OF TYPE I AND TYPE II FIBRES FROM MEDIAL AND LATERAL HEADS OF GASTROCNEMIUS

Post-mortem No.	*Type I (Medial–Lateral)* $(M_I–L_I)$	*Type II* $(M_{II}–L_{II})$	$(M_I–L_I) -(M_{II}–L_{II})$
1[a]			
2	27.9	23.5	4.4
3	13.9	14.3	−0.4
4	10.6	11.6	−1.0
5	21.0	19.8	1.2
6	3.5	−1.0	4.5
Mean difference	15.4	13.6	1.5
"t"$_4$ value	3.65	3.24	
Level of significance	$p<0.05$	$p<0.05$	

[a] In post-mortem No. 1, no sample of the medial head of gastrocnemius was taken so no comparison was possible.

DISCUSSION

The autopsy subjects from whom muscle samples were obtained probably constitute the most nearly normal and closely comparable group of individuals to be studied so far in respect of muscle fibre size. We restricted the age range of the subjects to little over one decade, taking into consideration the findings of Moore, Rebeiz, Holden and Adams (1971), who showed that mean fibre diameter varies with age, reaching a peak in the third or fourth decade of life and thereafter gradually decreasing. However, in spite of using a closely comparable group of individuals, the amount of variation in mean fibre size in any one muscle was considerable. Not only did the mean fibre sizes differ, but there were also striking variations in the *relative* sizes of Type I and Type II fibres in the same muscle in different subjects. The analysis of variance carried out on all muscle samples revealed this interaction effect between fibre type and individual post-mortem subject. As a consequence, the 95% confidence limits for the mean fibre diameter of Type I and Type II fibres in each muscle site reflect this wide variation (see Table 5).

It is not surprising, therefore, that the vast majority of "normal" data derived from previous studies fit neatly within the normal ranges as defined by the present study. A marginal exception to this is the mean size of Type II fibres in biceps brachii cited by Brooke and Engel (1969). Their figure of 72.6 μm falls just outside our own upper limit of normal and exceeds by more than 10 μm the mean value cited by Jennekens *et al.* (1971). It would perhaps be inappropriate to speculate on the reasons for this disparity between values obtained from a group of North American male subjects (Brooke and Engel 1969) and those derived from two groups of male subjects from the North of England (Jennekens *et al.* 1971 and the present study). However, one obvious factor to be considered is the inclusion in Brooke and Engel's survey of 1 particular subject who worked in a lumber yard and whom they admitted to have influenced

the final mean value disproportionately. This, of course, raises the whole question of the amount of heavy manual work done or strenuous exercise taken by individuals included in this type of study. In the present survey we had no precise data on this point, but could find no correlation between mean fibre diameter and the physical build of the subjects, which showed a certain amount of variation within the group (see Table 1).

Although there have been numerous papers dealing with muscle fibre size in human muscle, a large number of these were carried out on paraffin-embedded material. As a result, they include no data on the relative sizes of the two main histochemical fibre types. In the previous papers which do include data on this point, the accent has been placed rather on reporting a difference in the mean diameter of the two fibre types than on determining its statistical significance, if any. In this study, the overall mean diameter of Type II fibres was found to be greater than the mean Type I diameter in 44 out of the total of 50 sites. However, in only 15 cases was this difference a significant one. In very many cases an apparently large numerical difference in the mean size of the two fibre types proved to be non-significant because of the interaction effect discussed previously. Although it has been suggested that the tendency for Type II fibres to be larger than Type I fibres in male subjects as compared with female subjects is related to the work-load on particular muscles, we could find no evidence that those muscles involved in strenuous activity were more likely to show a significant difference in the size of the two fibre types than were postural muscles.

It was found that there was no significant difference in the mean diameters of either Type I or Type II fibres from surface and deep areas in the same muscle. However, there was a significant difference in the *relative* sizes of the two fibre types depending upon where they were located. The difference between the mean diameters of the fibre types was found to be greater in the deep areas than in the surface areas of those muscles in which multiple sites were sampled. When the mean fibre diameters were compared in the medial and lateral heads of the rectus femoris and in the clavicular and sternal heads of pectoralis major, it was found that there was no significant difference between the two heads of these muscles so far as fibre size was concerned. However, there was a significant difference between the medial and lateral heads of the gastrocnemius, the mean diameter of both fibre types being significantly larger in the medial head than in the lateral head. For this reason, it is recommended that when biopsy samples of this muscle are taken the exact site of the sample should be specified.

We could find no evidence that the diameter of Type II fibres was any more variable than that of Type I fibres within any one muscle. Moreover, the overall trend was for large Type I fibres to accompany large Type II fibres and for small Type I fibres to be found in conjunction with small Type II fibres. It therefore seems that the main way in which human muscles are adapted to fulfil their varying physiological roles is not by means of any constant or conspicuous difference in the sizes of the two fibre types, but rather by means of their varying numerical fibre type constitution.

ACKNOWLEDGEMENTS

We should like to thank Professor J. N. Walton for his helpful advice and criticism

throughout the course of this study. We are also grateful to Mrs. Valerie Giles, Miss Jennifer Allison and Mrs. Doreen Hargreaves for invaluable technical assistance.

SUMMARY

The mean diameters of Type I and Type II fibres were calculated in 50 muscle sites in each of 6 previously normal male autopsy subjects. Type II fibres were found to be larger than Type I fibres in 44 out of the total of 50 sites, but in only 15 sites was the difference statistically significant. Of the 6 muscle sites in which the mean diameter of Type I fibres exceeded that of Type II fibres, only one statistically significant difference was recorded. Significant differences in fibre size did not seem to be related to muscle function in any way.

Sampling multiple sites within one muscle revealed that the difference in the relative sizes of the two fibre types was greater in the deep areas than in the surface areas. The fibres of the medial head of gastrocnemius were found to be significantly larger than those of the lateral head; no such difference was found when the clavicular and sternal heads of pectoralis major and the lateral and medial heads of rectus femoris were compared.

Both fibre types seemed equally variable in respect of fibre size and, because of the considerable differences between post-mortem subjects the normal ranges of fibre size calculated for individual muscles are correspondingly large. Since the majority of muscles showed no significant difference in the diameters of the two fibre types, it was thought probable that human muscles are adapted for their different physiological roles by means of their widely varying fibre type constitution and that fibre size is relatively unimportant in this respect.

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Journal of the neurological Sciences, 1973, 20: 345–361

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A Comparison of Fibre Size, Fibre Type Constitution and Spatial Fibre Type Distribution in Normal Human Muscle and in Muscle from Cases of Spinal Muscular Atrophy and from Other Neuromuscular Disorders

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(Received 8 June, 1973)

INTRODUCTION

An obvious prerequisite in the study of any pathological condition is to obtain the best possible estimate of the limits of normality in comparable healthy individuals. We have found that there may be considerable variation in the mean size of muscle fibres and in the proportions of the two main histochemical fibre types in any one muscle when subjects free from any form of neuromuscular disease are compared, even though the subjects are of the same sex and of similar ages (Johnson, Polgar, Weightman and Appleton 1973; Polgar, Johnson, Weightman and Appleton 1973). The normal ranges of fibre size and of fibre type proportions derived from the study of such normal individuals have been used in this survey in order to determine the ways in which muscle samples from patients suffering from a variety of neuromuscular diseases may deviate from these normal values.

The main objective of this study has been to determine whether central denervating processes produce characteristic deviations in the numerical or spatial distribution of the two fibre types, or in fibre size, which are distinct, qualitatively or quantitatively, from those produced by other pathological entities. It is widely recognised that the histochemical type of a muscle fibre is dependent upon the nature of its nerve supply. Thus any change in the spatial distribution of the fibre types or a significant alteration in the overall fibre type constitution of a muscle would seem to presuppose either a physical or a physiological change in the innervation of the muscle. Studies on the normal spatial distribution of the fibre types have shown that a random pattern exists in almost all human muscles, and certainly in all those which are commonly biopsied (Johnson *et al.* 1973). The effects of the physical processes of denervation and reinnervation include the grouping of fibres of uniform histochemical type due to groups of denervated fibres becoming reinnervated by means of collateral branching from an axon or axons of one particular type. Uniform fibre type grouping has

thus become accepted as indicative of the occurrence of successive cycles of denervation and reinnervation (Engel 1965). However, the absence of uniform fibre type grouping cannot be held to preclude the occurrence of reinnervation in a given muscle. If, for example, the reinnervation of a group of denervated fibres is undertaken by axons of both types, then it is likely that a random distribution of fibres will result, in much the same way as it does in the initial process of innervation. It is therefore possible that reinnervation may produce a fibre type constitution which is significantly different from that of the previously normal muscle, while still preserving the usual random distribution of fibre types.

Since uniform fibre type grouping appears to involve changes in innervation pattern, one would expect this phenomenon to be confined to neuropathic disorders. However, it is obvious that a myopathic process, in which one particular fibre type is selectively affected by atrophy or necrosis, will ultimately result in an abnormal fibre type constitution in the affected muscle. Thus, a change in the fibre type proportions of a given muscle may be due either to a myopathic or to a neuropathic process.

In examining the nature of the changes in fibre size which are likely to occur in myopathic and neuropathic conditions, the classical concepts of these disorders allow one to form at least a tentative hypothesis. It is generally held that, in myopathic conditions, the involvement of individual muscle fibres is random, so that the atrophy or hypertrophy occurring in a myopathic process would be expected to increase the variance of a fibre population while preserving its unimodality. In a classic case of a disorder affecting whole motor units, however, one might reasonably expect to find a bimodal distribution of fibre sizes due to the fact that fibres of motor units affected by atrophy would differ markedly from residual fibres with normal diameters. This somewhat oversimplified picture would of course be modified by the occurrence of repeated cycles of denervation and reinnervation which would tend to blur the bimodality of the distribution. The presence of any "secondary myopathic change" in neuropathic disorders would also tend to have this effect.

In this study we have taken an unselected group of male patients, biopsied between 1969 and 1972, and have compared them with a group of previously normal autopsy subjects who were all young adult males. We felt justified in comparing only patients who fell within the same age range as that of the control group. In classifying patients into the various disease categories, we felt that every attempt should be made to do so on the basis of clinical, electrophysiological and biochemical findings without recourse to the histopathological diagnosis. The rationale underlying this approach was that, while we were trying to assess quantitatively the parameters of fibre size, fibre type constitution and spatial fibre type distribution, a qualitative assessment of these same parameters had participated, to a greater or lesser extent, in the formulation of the histological diagnosis.

MATERIALS AND METHODS

Muscle biopsy specimens were obtained from 19 patients aged between 16 and 33 years suffering from various forms of neuromuscular disease. In all cases serum en-

zyme estimations and detailed electromyographic (EMG) studies were performed. The diagnoses eventually reached in these 19 cases as a result of clinical, genetic and investigative findings will be described later. The site of muscle biopsy was vastus lateralis (10), deltoid (5) or biceps brachii (4). The muscle samples were trimmed to a size not exceeding 1 cm^3 and were frozen in dichloro-difluoro-methane (Arcton 12), cooled to $-150°C$ in liquid nitrogen. These biopsy samples were subjected to the usual histochemical examination and, in addition, the preparations stained to demonstrate the activity of myofibrillar ATP-ase (Hayashi and Freiman 1966) were photographed at a standard magnification for use in the estimation of fibre size and fibre type distribution. A random sample of 200 fibres from each biopsy was studied; the fibres were classified as Type I or Type II, and the diameters of these same 200 fibres were measured according to the method of Song, Shimada and Anderson (1963). In all respects, the biopsy samples were treated in exactly the same way as were the normal control samples used in the study, except that the latter were from autopsy subjects. All the autopsy muscle samples were taken within 24 hr of death, and it was found that there was no impairment of the histochemical reaction for myofibrillar ATP-ase in these specimens of muscle as compared with the biopsy samples.

The 6 autopsy subjects from whom normal control material was taken were aged between 17 and 30 years. The autopsy samples of biceps brachii, deltoid and vastus lateralis were taken as part of a much larger survey of normal fibre size and fibre type distribution (Polgar *et al.* 1973; Johnson *et al.* 1973). During these earlier studies we were also interested in finding a rapid method of assessing the normality or abnormality of the spatial distribution of the fibre types. In this objective we used the concept of the "enclosed fibre" originated by Jennekens, Tomlinson and Walton (1971). An "enclosed fibre" is defined as a fibre which is completely surrounded by fibres of its own fibre type. Thus, as the proportion of a given fibre type within a population increases, the number of "enclosed fibres" will also increase, assuming a random distribution of the fibre types.

The arrangement of muscle fibres in fasciculi approximates to a hexagonal lattice so that the probability of a Type I fibre being surrounded by six other Type I fibres will be p^6, where p is the proportion of Type I fibres in the sample. In a sample of N fibres the expected number of enclosed Type I fibres will be $N_p 7$ and since the number of enclosed fibres is binomially distributed the standard deviation can be expressed as $\sqrt{N_p 7(1-p^6)}$. It is therefore possible to predict the number of enclosed fibres expected in any sample of known fibre type constitution. A significantly increased number of enclosed fibres is indicative of an abnormal degree of uniform fibre type grouping and thus the estimation of the number of enclosed fibres is a convenient way of detecting any departure from the normal random arrangement of the fibre types.

BIOCHEMICAL INVESTIGATIONS

Serum creatine kinase levels were estimated according to the method of Hughes (1962) modified by Pearce, Pennington and Walton (1969).

EMG investigations

Routine concentric needle electromyography was performed in all the patients by a number of different but experienced observers (see ACKNOWLEDGEMENTS). The presence of any spontaneous activity or abnormal insertional activity was noted. The nature of volitional activity in terms of interference pattern and the amplitude and duration of motor unit potentials was recorded. In the great majority of cases, motor nerve conduction studies were also carried out. Estimations of the number of motor units in extensor digitorum brevis (McComas, Fawcett, Campbell and Sica 1971) and sensory nerve conduction studies were made in about 50% of the patients.

RESULTS

The ages of the patients studied in this survey are listed in Table 1 together with the muscle sites from which biopsies were obtained. In 1 instance (patient W.A.), 2 biopsies were taken, the first from the right and the second from the left vastus lateralis after an interval of 6 months.

TABLE 1

THE AGES AT THE TIME OF BIOPSY AND BIOPSY SITES OF THE PATIENTS SUFFERING FROM VARIOUS FORMS OF NEUROMUSCULAR DISEASE

Patient	*Age at time of biopsy (years)*	*Site of biopsy*
J.R.	16	biceps brachii
D.H.	32	biceps brachii
A.W.	30	biceps brachii
G.M.	17	biceps brachii
B.G.	25	deltoid
D.P.	24	deltoid
K.W.	32	deltoid
J.Mc.	19	deltoid
T.B.	28	deltoid
B.L.	26	vastus lateralis
F.T.	33	vastus lateralis
A.S.	16	vastus lateralis
F.S.	26	vastus lateralis
W.G.	29	vastus lateralis
I.S.	17	vastus lateralis
J.H.	17	vastus lateralis
N.H.	21	vastus lateralis
J.M.	16	vastus lateralis
W.A.	30	vastus lateralis
W.A.	31	vastus lateralis

Table 2 summarises the clinical assessment and the electrophysiological and biochemical findings which contributed to the diagnoses of the patients' disorders. As we have stated previously, it was felt that the histopathological findings should be excluded at this stage from such an assessment, but these were used later as a check on the resulting classification. In 4 cases, the EMG and histological findings were found to be at variance. In patient J.R. the presence of myopathic features, including

TABLE 2

SUMMARY OF THE CLINICAL, ELECTROPHYSIOLOGICAL AND BIOCHEMICAL FINDINGS CONTRIBUTING TO A DIAGNOSIS

Patient	*Initial clinical diagnosis*	*EMG findings*	*Serum creatine kinase activities*[a]	*Final diagnosis*
J.R.	spinal muscular atrophy	chronic denervation	50 *N*	spinal muscular atrophy
D.H.	limb-girdle dystrophy or spinal muscular atrophy	diffuse loss of motor units	*N*	spinal muscular atrophy
A.W.	spinal muscular atrophy	myopathic; motor unit count only mildly abnormal	24 *N*	proximal myopathy
G.M.	myopathy	severe myopathic disorder maximum in proximal muscles	*N* (on prednisone)	proximal myopathy
B.G.	spinal muscular atrophy	possibility of chronic partial denervation	2 *N*	spinal muscular atrophy
D.P.	spinal muscular atrophy	severe loss of motor units	3 *N*	spinal muscular atrophy
K.W.	limb-girdle dystrophy or spinal muscular atrophy	myopathic with polyphasic potentials	13 *N*	spinal muscular atrophy
J.Mc.	limb-girdle dystrophy or spinal muscular atrophy	severe generalised myopathy	40 *N*	limb-girdle dystrophy
T.B.	myotonic dystrophy	typical features of myotonia	1.5 *N*	myotonic dystrophy
B.L.	Becker dystrophy or limb-girdle dystrophy	myopathic features in upper and lower limbs	20 *N*	Becker dystrophy
F.T.	spinal muscular atrophy	chronic denervation with secondary myopathic change	70 *N*	spinal muscular atrophy
A.S.	spinal muscular atrophy	active denervation with chronic loss of motor units	2 *N*	spinal muscular atrophy
F.S.	limb-girdle dystrophy or spinal muscular atrophy	generalised chronic loss of motor units	8 *N*	spinal muscular atrophy
W.G.	facio-scapulo-humeral dystrophy	mixed myopathic and neuropathic features	13 *N*	facio-scapulo-humeral dystrophy
I.S.	spinal muscular atrophy	suggestive of chronic denervation but no active neuropathy	1.5 *N*	spinal muscular atrophy
J.H.	limb-girdle dystrophy or spinal muscular atrophy	chronic denervation, pseudomyotonia, and some myopathic changes	43 *N*	spinal muscular atrophy
N.H.	spinal muscular atrophy	chronic denervation with secondary myopathic change	10 *N*	spinal muscular atrophy
J.M.	Becker dystrophy or spinal muscular atrophy	consistent with chronic proximal denervation	65 *N*	spinal muscular atrophy
W.A.	polymyositis	compatible with a myopathic process	65 *N*	polymyositis

[a] Serum creatine kinase activities are expressed as multiples of the upper limit of normal; $N = 60$ IU/l.

perivascular lymphocytic infiltration, was sufficiently marked to be interpreted as constituting a myositis whereas the EMG findings were strongly suggestive of a central denervating process. The very high serum creatine kinase activity in this patient would seem to reflect the severity of muscle necrosis, but even higher levels have been noted in cases in which the diagnosis of spinal muscular atrophy was unequivocal *e.g.* patient F.T. In patient K.W. the presence of gross "secondary myopathic change" was probably responsible for the discrepancy between the EMG findings which were exclusively myopathic on the one hand and the histological picture on the other, which indicated an underlying denervating process. A similarly

conflicting picture was seen in the case of J. Mc., illustrating the particular difficulties inherent in trying to distinguish between cases of spinal muscular atrophy with severe secondary myopathic change and genuine cases of limb-girdle dystrophy. In patient I.S. the histochemical findings included mild mitochondrial abnormalities, but although no histological indication of a denervating process was found, there was nothing to conflict with the EMG diagnosis of a mild central denervating process.

In patient G.M. histological examination confirmed the presence of a myopathic process, and subsequent histochemical investigation suggested that this was due to a defect in lipid metabolism. Similarly, in patient A.W., the myopathic features detected on EMG examination could be correlated with the gross mitochondrial abnormalities which were detected histochemically. In the remaining patients, histological and histochemical findings were corroborative but did not yield any additional information of diagnostic significance.

The serum creatine kinase activities were raised in almost all the patients. In cases of spinal muscular atrophy uncomplicated by secondary myopathic change, the level is unlikely to exceed 5 times the normal value (Dr. M. J. Campbell, personal communication). However, in many of the patients included in this survey, where the diagnosis of spinal muscular atrophy was confirmed, the serum enzyme levels were greatly in excess of this, due presumably to the prevalence of secondary myopathic change in the patients thus affected.

In general, the results of the biochemical and EMG investigations were compatible with the initial clinical diagnosis. The EMG studies were undoubtedly of prime importance in distinguishing between cases of spinal muscular atrophy and those with late-onset muscular dystrophy; in many such cases the clinical diagnosis could be only tentative.

In this survey, where we were primarily concerned with the presence or absence of characteristic differences in fibre size and fibre type distribution between "neuropathic" and "myopathic" disorders, we aimed at an initially simple classification of cases into these two categories. However, because of the reasons outlined above, a proportion of the cases would be more readily accommodated in an intermediate category comprising mixed myopathic and neuropathic elements.

The group of "neurogenic" disorders consists of either confirmed or probable cases of spinal muscular atrophy. The "myopathic" group, however, is totally heterogeneous, comprising 1 case each of limb-girdle dystrophy, facio-scapulo-humeral dystrophy, Becker dystrophy, 2 cases of metabolic myopathies involving lipid and mitochondrial disorders respectively (G.M. and A.W.), a case of polymyositis from which two biopsies were obtained, and a single case of myotonic dystrophy. The heterogeneity of this group was felt to be advantageous in some respects; in examining the group of spinal muscular atrophy patients for features which were solely characteristic of them as a group, it was obviously important to have a broad selection of myopathic disorders with which to compare them.

Fibre type distribution and fibre sizes in the normal control muscles

The mean values for the Type I fibre proportions in biceps brachii, deltoid and vastus lateralis in the control subjects are given in Table 3, with the corresponding

TABLE 3

TYPE I FIBRE % IN THE 6 NORMAL AUTOPSY SUBJECTS, IN BICEPS BRACHII, DELTOID AND VASTUS LATERALIS

	Post mortem No.						*Mean*	*Normal range*[a]
	1	*2*	*3*	*4*	*5*	*6*		
Biceps brachii	53.3	34.4	34.4	49.0	45.0	37.4	42.3	33.9–50.7
Deltoid	67.6	62.1	47.9	49.8	43.1	49.1	53.3	43.4–63.2
Vastus lateralis	45.5	33.8	41.4	49.8	23.3	32.9	37.8	27.7–47.9

[a]Based on mean ±2.57 S.D.

normal ranges for these values. Due to the relatively large amount of variation in fibre type proportions within this normal group, it will be seen that there is a range of approximately 20% between the upper and lower limits for all 3 muscles.

In the biceps brachii, deltoid and vastus lateralis of the normal subjects it was found that the observed numbers of "enclosed" fibres of both fibre types were in accordance with the numbers to be expected on the basis of a random distribution of the two fibre types. There was thus no evidence of uniform fibre type grouping in the control material. Table 4 sets out the expected number of enclosed fibres for a complete range of fibre type proportions. For example, if, in the sample of 200 fibres, 160 (80%) are of one particular type, one would expect to find, in 95 cases out of 100, between 31 and 52 "enclosed fibres" of that type in the sample, if in fact the fibre types were randomly distributed. Any significant increase in the number of enclosed fibres can be taken to indicate the presence of uniform fibre type grouping.

The normal mean values for the diameters of Type 1 and Type II fibres in biceps

TABLE 4

THE RELATIONSHIP BETWEEN THE OBSERVED NUMBER OF MUSCLE FIBRES OF A PARTICULAR FIBRE TYPE AND THE EXPECTED NUMBER OF "ENCLOSED" FIBRES OF THAT TYPE

No. of fibres of given type in sample of 200 fibres	*Expected no. "enclosed" fibres of that type*	*95% confidence limits*
0	0.0	0–0
20	0.0	0–0
40	0.0	0–0
60	0.0	0–0
80	0.3	0–1
100	1.6	0–4
120	5.6	1–10
140	16.5	9–24
160	41.9	31–52
170	64.1	52–76
180	95.7	83–109
190	139.7	128–151
195	167.5	158–177
200	200.0	200–200

TABLE 5

THE MEAN DIAMETERS OF TYPE I AND TYPE II FIBRES IN THE 6 NORMAL AUTOPSY SUBJECTS IN BICEPS BRACHII, DELTOID AND VASTUS LATERALIS

	Type I fibres (μm)	*Normal range*	*Type II fibres (μm)*	*Normal range*
Biceps brachii	50.5	31–70	55.8	31–72
Deltoid	51.6	33–70	58.6	27–90
Vastus lateralis	63.1	40–87	63.2	48–78

Normal range based on Mean ±2.57 S.D.

TABLE 6

PERCENTAGE OF TYPE I FIBRES IN MUSCLES FROM PATIENTS SUFFERING FROM NEUROMUSCULAR DISEASE

Muscle	*Patient*	*Type I fibres (%)*	*Normal range*
Biceps brachii	J.R.	46.0	22–63%
	D.H.	83.0↑	
	A.W.	90.5↑	
	G.M.	60.5	
Deltoid	B.G.	97.0↑	30–76%
	D.P.	85.5↑	
	K.W.	88.0↑	
	J.Mc.	75.5	
	T.B.	21.5↓	
Vastus lateralis	B.L.	39.0	13–63%
	F.T.	27.5	
	A.S.	0.0↓	
	F.S.	67.0↑	
	W.G.	47.0	
	I.S.	51.5	
	J.H.	27.5	
	N.H.	57.5	
	J.M.	44.0	
	W.A. (i)	74.0↑	
	W.A. (ii)	37.5	

↑denotes a significantly raised proportion of Type I fibres ($P < 0.05$).
↓denotes a significantly lowered proportion of Type I fibres ($P < 0.05$)

brachii, deltoid and vastus lateralis are listed in Table 5 which also sets out the normal ranges for these mean values.

Fibre type distribution and fibre sizes in muscle biopsies from cases of neuromuscular disease

The fibre type proportions in the biopsy samples of 200 fibres were calculated and are given in Table 6 with the normal ranges for the control subjects for comparison. Because there is a considerable degree of variation in fibre type proportion between normal subjects, these normal ranges are correspondingly wide. Even so, the numeri-

cal fibre type constitutions of almost half the biopsies were outside these limits. Nine biopsies out of the total of 20 showed grossly abnormal fibre type proportions; in 7 biopsies the percentage of Type I fibres was significantly raised, and in the other 2 it was significantly lowered. In the remaining 11 biopsies the proportions of the fibre types were within normal limits.

As well as examining the numerical distribution of the fibre types, their spatial distribution was studied, by counting the number of "enclosed fibres" of each fibre type in each sample and relating this to the numerical fibre type proportion. The observed number of "enclosed fibres" was compared with the number expected, assuming a random distribution of the fibres of each type. The results of the comparison of observed and expected numbers of enclosed fibres are given in Table 7. It was found that the great majority of the observed values for the numbers of enclosed fibres were in accordance with the expected values. However, in the cases indicated, a raised number of enclosed fibres was found to be statistically significant and thus provided evidence of a departure from a random distribution of the fibre types. In 1 case (patient N.H.) both fibre types showed this abnormal spatial distribution, but in the other 4 cases this evidence of uniform fibre type grouping was confined to only one fibre type.

TABLE 7

COMPARISON OF THE NUMBER OF ENCLOSED FIBRES OBSERVED IN SAMPLES OF MUSCLE FROM PATIENTS SUFFERING FROM NEUROMUSCULAR DISEASE, AND THE NUMBERS EXPECTED IN NORMAL RANDOM POPULATIONS

Muscle	*Patient*	*Type I*			*Type II*		
		N_1	E_1	O_1	N_2	E_2	O_2
Biceps brachii	J.R.	92	0.9	0	108	2.7	2
	D.H.	166	54.3	42	34	0.0	0
	A.W.	181	99.4	137↑	19	0.0	0
	G.M.	121	5.9	0	79	0.3	0
Deltoid	B.G.	194	161.6	154	6	0.0	0
	D.P.	171	66.8	70	29	0.0	0
	K.W.	176	81.7	112↑	24	0.0	0
	J.Mc.	151	28.0	47↑	49	0.0	1
	T.B.	43	0.0	0	157	36.7	45
Vastus lateralis	B.L.	78	0.3	0	122	6.3	10
	F.T.	55	0.0	0	145	21.1	21
	A.S.	0	0.0	0	200	200	200
	F.S.	134	12.1	17	66	0.1	0
	W.G.	94	1.0	1	106	2.3	1
	I.S.	103	1.9	0	97	1.3	0
	J.H.	55	0.0	0	145	21.1	64↑
	N.H.	115	4.2	24↑	85	0.5	28↑
	J.M.	88	0.6	0	112	3.5	5
	W.A. (i)	148	24.3	26	52	0.0	0
	W.A. (ii)	77	0.3	0	123	6.7	4

N_1, N_2: No. of Type I, Type II fibres in the samples.
E_1, E_2: expected number of enclosed Type I, Type II fibres in the sample.
O_1, O_2: observed number of enclosed fibres.
↑ denotes significantly raised number of enclosed fibres ($P < 0.001$).

TABLE 8

COMPARISON OF FIBRE DIAMETERS IN PATIENTS WITH NEUROMUSCULAR DISEASE AND IN NORMAL CONTROL SUBJECTS

Muscle	*Patient*	*Type I*		*Type II*	
		Mean (μm)	*Co. var.*	*Mean (μm)*	*Co. var.*
Biceps brachii	J.R.	58.7	22.8*	66.0	30.2*
	D.H.	61.6	49.2*	71.8	54.2*
	A.W.	98.0↑	34.1*	60.8	53.3*
	G.M.	34.8	26.7*	51.8	11.8
Normal ranges: Type I mean 31–70 μm; Type II mean 31–72 μm Type I Co. var. 12.4–18.8; Type II Co. var. 9.6–22.9					
Deltoid	B.G.	99.7↑	36.6*	53.3	37.0*
	D.P.	90.7↑	32.3*	48.0	67.1*
	K.W.	82.3↑	40.0*	44.3	58.7*
	J.Mc	65.2	32.5*	69.0	46.7*
	T.B.	53.8	17.7	78.6	14.1
Normal ranges: Type I mean 33–70 μm; Type II mean 27–90 μm Type I Co. var. 9.8–24.4; Type II Co. var. 10.2–23.3					
Vastus lateralis	B.L.	83.3	30.6*	83.4↑	40.9*
	F.T.	58.2	23.9*	52.4	29.8*
	A.S.	—	—	85.4↑	17.7
	F.S.	70.6	38.0*	63.6	49.4*
	W.G.	107.4↑	30.6*	104.9↑	30.3*
	I.S.	56.2	12.8	48.6	17.9
	J.H.	56.9	56.9*	66.2	58.0*
	N.H.	34.6↓	40.8*	57.5	41.0*
	J.M.	55.6	63.8*	67.1	68.0*
	W.A. (i)	48.9	41.1*	56.6	50.7*
	W.A. (ii)	72.5	29.9*	68.6	28.0*
Normal ranges: Type I mean 40–87 μm; Type II mean 48–78 μm Type I Co. var. 12.0–19.1; Type II Co. var. 13.2–22.6					

$$\text{Coefficient of variation (Co. var.)} = \frac{100 \text{ standard deviation}}{\text{mean}}.$$

↑ mean fibre diameter significantly raised ($P < 0.05$).
↓ mean fibre diameter significantly decreased ($P < 0.05$).
* coefficient of variation above normal limits.

The fibre diameters from the biopsy samples were compared with the normal values derived from the 6 autopsy subjects (Table 8). We were interested not only in the normality or otherwise of the mean fibre diameters in the patients' biopsies, but also in the nature of the fibre size distribution. To some extent, the direct examination of the coefficients of variation will reveal features such as any large increase in the spread of the size distribution, but will not, of course, give any indication as to the unimodal or bimodal nature of the distribution. In order to assess this type of variation in distribution, it is necessary to construct histograms; these are shown in Figs. 1 and 2, with the control histograms for biceps brachii, deltoid and vastus lateralis placed in apposition for comparison. It will be seen that all the samples of

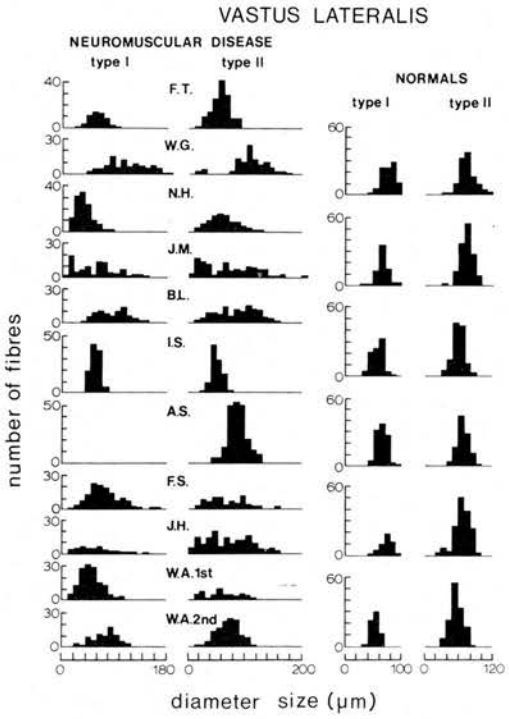

Fig. 1. The size distributions of Type I and Type II fibres in normal and pathological samples of vastus lateralis.

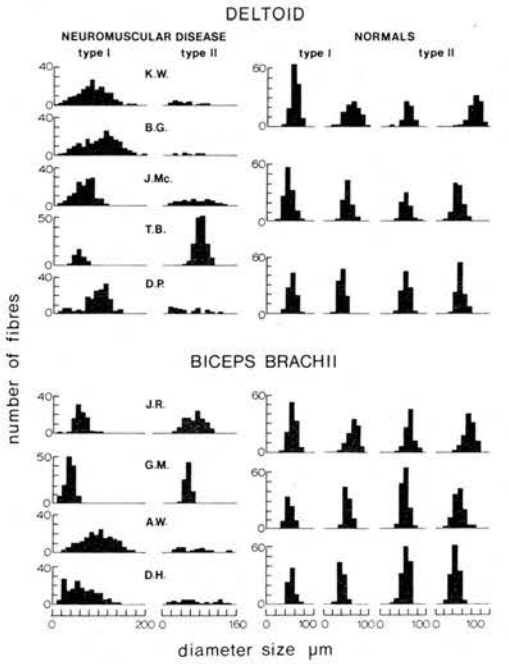

Fig. 2. The size distributions of Type I and Type II fibres in normal and pathological samples of deltoid and biceps brachii.

normal muscle proved to be unimodal as far as both Type I and Type II fibres are concerned. There was evidence of both positive and negative skewness in some of the distributions of diameter sizes for both normal control material and the patients' biopsies. This evidence was present in all three muscles and in both fibre types. Since some skew distributions were found in normal subjects, the skewness of some of the patients' histograms was held to be not significant.

From Table 8, it can be seen that the range of fibre sizes was increased above the normal values in all except 3 of the biopsies. In contrast, relatively few values for the mean fibre diameter were outside the normal limits, a net increase in fibre diameter being a more common finding than net atrophy. It is apparent, in such muscles, that the wasting which is observed clinically is more likely to be due to actual fibre loss than to a net decrease in average fibre diameter. Examination of the individual histograms emphasised the fact that few cases showed a distribution of fibre sizes similar to that of the control subjects. Those considered to be within normal limits were patients T.B. and I.S., though the mean fibre size of Type II fibres in the latter case was in fact minimally decreased. In patient G.M. the Type II fibres were within normal limits, but the coefficient of variation of the Type I fibres was outside the normal range. From the point of view of fibre size alone, patient A.S. appeared to

TABLE 9

SUMMARY OF THE OVERALL CHARACTERISTICS OF FIBRE TYPE DISTRIBUTION AND FIBRE SIZE IN PATIENTS WITH NEUROMUSCULAR DISEASE

	Patient	*Type I (%)*	*Enclosed fibres*		*Fibre diameter*		*Coefficient of variation*	
			Type I	*Type II*	*Type I*	*Type II*	*Type I*	*Type II*
Neuropathic disorders	B.G.	↑			↑		*	*
	N.H.		↑	↑	↓		*	*
	A.S.	↓	–		–	↑	–	
	D.P.	↑			↑		*	*
	D.H.	↑					*	*
	F.S.	↑					*	*
	J.H.			↑			*	*
	F.T.						*	*
	J.M.						*	*
	K.W.	↑	↑		↑		*	*
	I.S.							
	J.R.						*	*
Myopathic disorders	J.Mc.		↑				*	*
	A.W.	↑	↑		↑		*	*
	W.G.				↑	↑	*	*
	T.B.	↓						
	B.L.					↑	*	*
	G.M.						*	
	W.A. (i)	↑					*	*
	W.A. (ii)						*	*

↑ denotes value significantly raised.
↓ denotes value significantly lowered.
* denotes significant increase in variance.

have a distribution remarkably similar to that of the control subjects, but this case was characterised by a complete absence of Type I fibres in the random area of 200 fibres which was examined.

There were no unequivocal instances of a bimodal distribution in the abnormal biopsies. In those patients in which numerous atrophied or hypertrophied fibres were found, these abnormal fibres did not constitute distinct fibre populations but merely formed the limits of an increased spectrum of fibre sizes.

A summary of the overall characteristics of the numerical and spatial fibre type distributions and of the fibre size distributions in the 20 biopsies as compared with the normal controls is given in Table 9. In this table, we have separated the biopsies on the basis of whether the patients were considered to be suffering from neuropathic or myopathic disorders (see Table 2), in order to be able to appreciate more readily any features which might prove to be solely characteristic of the neuropathic group.

DISCUSSION

It has already been postulated on theoretical grounds that a significant alteration in fibre type constitution may well occur in neuropathic disorders due to the effects of denervation and reinnervation. However, the cases of spinal muscular atrophy studied in this survey included some in which the fibre type constitution was within normal limits or else only marginally abnormal. Moreover, the findings within the group of "myopathic" disorders plainly indicated that reinnervation is by no means the only mechanism responsible for observed alterations in fibre type constitution.

The participation of a neurogenic factor in the pathogenesis of myotonic dystrophy as described by McComas, Campbell and Sica (1971) is not disputed, but there is no evidence to suggest that reinnervation of atrophied fibres takes place to any significant extent. It is much more probable that the abnormally high proportion of Type II fibres seen in patient T.B. is due simply to the selective atrophy of Type I fibres which is a well known characteristic of the disease (Brooke and Engel 1969). The raised Type I fibre proportions in patient G.M. (lipid storage myopathy) and patient W.A. (polymyositis) are much less readily explained. However, it is interesting to note that a second biopsy from W.A. after approximately 6 months' steroid therapy showed that the fibre type constitution in the vastus lateralis of the collateral limb was within normal limits.

There is a growing body of evidence to suggest that feedback mechanisms from the muscle itself may profoundly influence the nature of the nerve impulse along the axons supplying the muscle. For example, the excision of synergistic muscles has been observed to transform the activity of rat plantaris and soleus muscles into a much slower pattern of activity (Guth and Yellin 1971). This change, which involved an increase in the proportion of Type I ("slow") fibres, was postulated to occur as a result of the altered function of the muscles, which were now more involved in weight-bearing and less concerned in phasic contraction than hitherto. It seems reasonable to suppose that a similar shift in the physiological and histochemical profile of a muscle may occur when its normal functions are restricted and modified by disease. In the experimental study in the rat (Guth and Yellin 1971) it was stressed that the

physical innervation pattern of the muscle was not altered in any way; in myopathic conditions where the occurrence of reinnervation can be discounted with reasonable certainty, changes in fibre type constitution may well be due to the operation of similar feedback mechanisms. Within the group of "myopathic" patients included in this survey, any shift in fibre type constitution which occurred was, in fact, in the direction which would result in a slowing of the muscle (*i.e.* an increase in the proportion of Type I fibres), with the exception of the case of myotonic dystrophy in which the mechanism underlying the shift in fibre type constitution is more obvious and certainly different from the other myopathic cases.

Examination of the spatial fibre type distribution of the muscle samples yielded relatively few instances in which a significantly raised number of "enclosed fibres" was recorded. It was expected that a high proportion of the cases of spinal muscular atrophy which were included in the survey would show some evidence of uniform fibre type grouping; in fact, only 3 cases of spinal muscular atrophy (N.H., J.H. and K.W.) were affected in this way. It has been stated earlier that reinnervation will only result in uniform fibre type grouping if certain criteria are first satisfied, namely that the group of atrophied fibres to be reinnervated should be compact, and that the reinnervating axons should all be of one type. It is possible, therefore, that these conditions were not fulfilled in the muscle samples examined or, more simply, that reinnervation was not a striking feature of the disease process at that particular point in time.

The technique used to estimate the amount of uniform fibre type grouping is designed to distinguish between an increase in the number of enclosed fibres which occurs to a degree commensurate with an increase in the numerical proportion of that fibre type and an increase in the number of enclosed fibres which occurs independently of any shift in fibre type constitution. Thus in the case of patient A.S., in which 100 % Type II fibres were recorded in the sample studied, the number of enclosed fibres was inevitably dependent upon this factor. It is obvious that such a dramatic change in fibre type constitution cannot be explained on any other basis than that of reinnervation, but in cases where the shift in fibre type constitution is less marked, it is useful to have some corroborative evidence that reinnervation has occurred. It was precisely in this connection that we became interested in estimating the number of enclosed fibres which gives a simple indication of the randomness or non-randomness of a fibre population. However, it does appear that the use of uniform fibre type grouping as a definite index of reinnervation may not after all be justified. Two patients included in the "myopathic" group in the present survey showed evidence of uniform fibre type grouping. Admittedly the cases (patients J.Mc. and A.W.) may be regarded as more properly belonging to a class in which the mechanism underlying the disease may well affect both muscle and nerve. However, the EMG studies performed on J.Mc. gave evidence of a severe generalised myopathy only; those carried out on A.W. showed features of a myopathy rather than a neuropathy, with a motor unit estimate in the extensor digitorum brevis (McComas *et al.* 1971) which was only mildly abnormal.

It is possible to rationalise the occurrence of uniform fibre type grouping in myopathic conditions on two grounds. Firstly, a selective process may result in the com-

plete loss of one fibre type from restricted areas of the muscle. However, according to theoretical considerations, this type of myopathic process should not have the effect of altering the spatial fibre type distribution from random to non-random, since the essence of a myopathic process is that it affects individual muscle fibres randomly. The second, and more likely, possibility is that, in a necrotising myopathy, regenerating fibres are frequently found to be disposed in groups in the vicinity of a former necrotic fibre or fibres. If such regenerating fibres reach maturity, they will presumably receive their innervation from the collateral sprouting of the most nearly adjacent axons. In this way, muscle regeneration may well give rise to clusters of uniform type fibres in a way which provides an obvious parallel to the reinnervation of grouped atrophied fibres.

Although an increase in the range of fibre size was an almost universal feature of the muscles studied, this gave rise only occasionally to a significant deviation in the mean fibre diameter. This was true both of the "neuropathic" and "myopathic" groups. An interesting finding was that of the 8 cases showing a shift in mean fibre diameter, only 1 showed a decrease; in the other cases, hypertrophied fibres obviously more than compensated for atrophied fibres, resulting in a net increase in the mean diameter. It is evident in such cases that the muscle wasting which is apparent clinically must be due to actual fibre loss. In 1 patient (A.S.) the process of reinnervation appeared to be extremely efficient over large areas of the biopsy. Histochemically, all the fibres sampled were Type II, but routine histological examination of the same area would undoubtedly have failed to detect any abnormality since the range of fibre size was within normal limits and the moderate degree of generalised hypertrophy was not apparent until quantitative studies were carried out.

Definite bimodal size distributions were not found in any of the biopsies studied. These were not expected in the "myopathic" disorders, where the random nature of the process should merely lead to an increase in the variance of the fibre population. However, in the group of spinal muscular atrophy patients, it was thought that atrophy of individual motor units in an otherwise normal fibre population might lead to such a distribution. This may well occur in more acute forms of spinal muscular atrophy such as Werdnig–Hoffman disease, but in the group of patients in this survey one of the effects of the secondary myopathic change, which was present in some degree in almost all the patients, may have been to mask any evidence of it.

The practical problems involved in distinguishing cases of some of the late-onset dystrophies from cases of spinal muscular atrophy are sometimes such that they defy the present methods of electrophysiological, biochemical and histological assessment. In analysing a group of biopsies from spinal muscular atrophy patients, in respect of fibre size distribution and spatial and numerical fibre type distribution, it was hoped that at least some features might emerge which were solely characteristic of the group and which would therefore be of diagnostic use. However, the results of this analysis did not yield such evidence. In fact, they showed that each of the abnormalities of fibre size and fibre type distribution found in the spinal muscular atrophy group can also be encountered in biopsies from patients suffering from muscle disorders other than those involving primary denervating processes.

ACKNOWLEDGEMENTS

We should like to thank Professor J. N. Walton for his continued interest and advice during this study. We are indebted to Dr. P. Hudgson and Dr. W. G. Bradley for access to clinical material and for their helpful criticism of the manuscript. The EMG investigations were performed by Dr. M. J. Campbell, Dr. A. R. M. Upton, Dr. D. D. Barwick, Dr. D. Gardner-Medwin and Dr. J. C. Brown. The biochemical studies were carried out by Dr. R. J. Pennington and Mrs. M. Parsons.

SUMMARY

Muscle biopsies from 19 patients suffering from various forms of neuromuscular disease were analysed in respect of fibre size and spatial and numerical fibre type distribution. The normal ranges of fibre size and fibre type constitution with which they were compared were derived from the corresponding muscles of 6 previously normal autopsy subjects who were of the same age and sex as the patients included in the survey. The patients were divided into two groups, those suffering from central denervating disorders and those suffering from predominantly myopathic diseases, in order to determine whether there were any quantifiable abnormalities of fibre type distribution which were solely characteristic of the former group.

The numerical fibre type constitution was found to be outside normal limits in almost half the patients' biopsies, whether they were affected by denervating processes or not. Conversely, relatively few cases of actual uniform fibre type grouping were encountered. The possible mechanisms, other than reinnervation, which may lead to alterations in fibre type proportions or in spatial fibre type distribution, are discussed.

In respect of fibre size distribution, almost all the biopsies showed evidence of an increase in the variance of the fibre population, but few instances of significant increase or decrease in mean diameter were recorded. Hypertrophy of a proportion of the fibre population frequently compensated for any atrophy which might occur, and this exerted a normative effect on the mean fibre diameter. It was concluded that the muscle wasting observed clinically, both in spinal muscular atrophy and in late-onset dystrophies, is more likely to be due to actual fibre loss than to a net decrease in average fibre diameter.

No abnormalities of fibre size or fibre type distribution were found which were entirely specific to central denervating disorders.

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Sarah Appleton

2. Digitisation of M-Mode echocardiography. Its reproducibility and sources of imprecision

Royal Victoria Infirmary Study:
R. E. Bullock, H. Amer, C. Albers, D. Appleton and R. Hall
Freeman Hospital Study:
K. Jennings, C. Griffiths, K. Evemy, A. Austin, A. Murray and S. Hunter

INTRODUCTION

The digitisation technique

M-Mode echocardiography is established as a safe and valuable diagnostic aid. Much of the information it provides is qualitative and heavily dependent on the skill and experience of the operator to recognise intracardiac structures and to make an assessment of wall movement. However, cardiac dimensions can be quantitated and measurements made of rates of movement of intracardiac structures. Although simple measurements, such as dimension, can be obtained with a ruler, echocardiograms lend themselves to computer processing which can be used to calculate ventricular dimensions and their rates of change with time continuously throughout the cardiac cycle. On-line image analysis systems transfer information direct to the computer without the need for recording, and store the data for later analysis (Hirsch et al 1973; Emerson et al 1977). This requires a large amount of data processing, fast analogue to digital conversion, large storage capacity and complicated pattern recognition algorithms to distinguish intracardiac structures. Off-line analysis, or digitisation, allows for human interpretation of cardiac echoes which remains the most accurate form of pattern recognition and also permits human quality control and is thereby cheaper and simpler.

Using the latter system, recordings are processed by deriving a series of co-ordinates representing the position of the echoes to be studied throughout the cardiac cycle. This is achieved by placing the record on a digitising tablet and tracing each echo of interest by hand with a cursor whose position is detected and converted to digital co-ordinates for further processing by the computer. The principles of this procedure have been described by Gibson and Brown in 1973. A digitising programme has been developed in Newcastle (Griffiths et al, 1982) which uses an 'Apple' microcomputer and a digitising tablet. This system was used for the studies described in this chapter.

Co-ordinates can be generated from echoes arising from either septal surface, the endo or epicardial aspect of the posterior wall or any continuous signal such

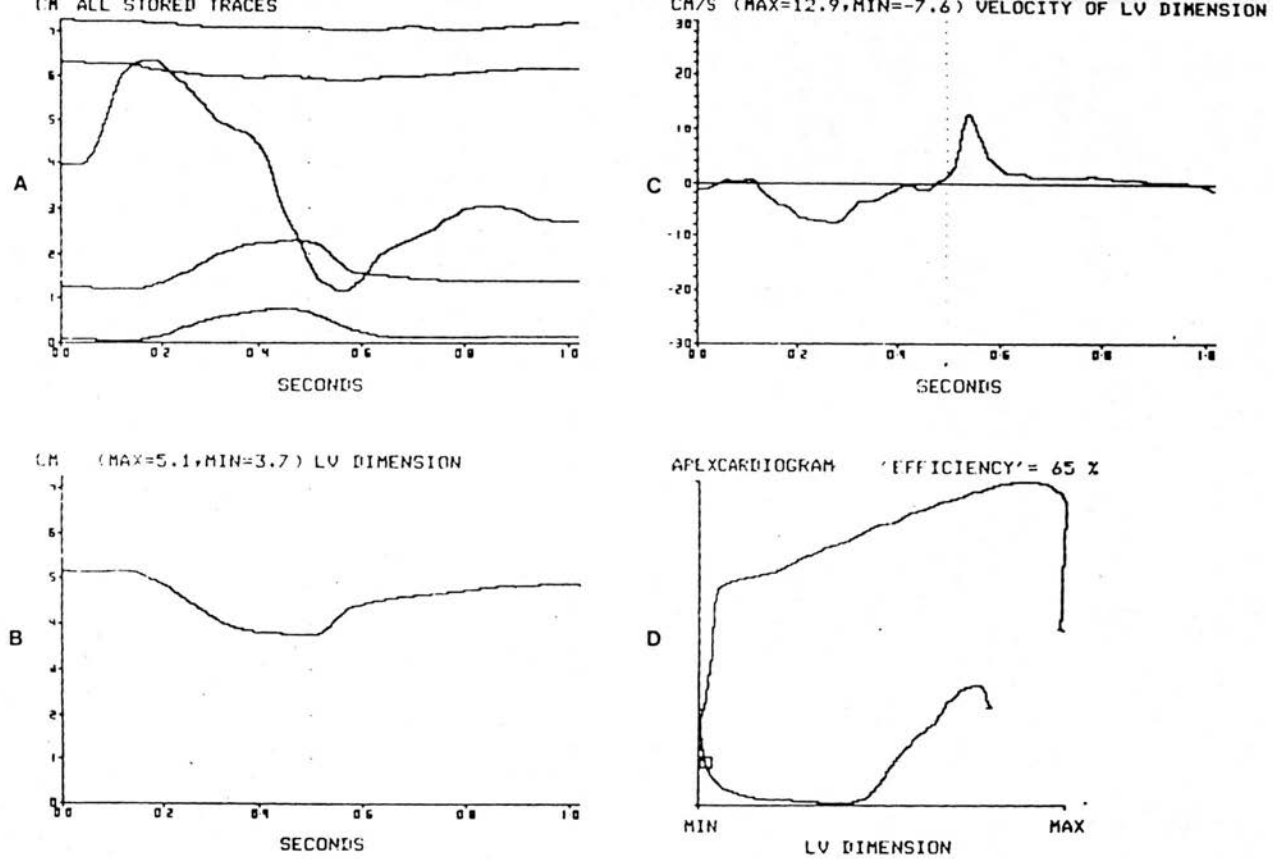


Fig. 2.1 Output generated by the Newcastle EMMA system (a) stored traces; (b) LV dimension; (c) rate of change of LV dimension: (d) apex-cardiogram — LV dimension 'loop'. Maximum and minimum values are shown where appropriate. The 'loop efficiency' is calculated as the ratio of the area of the loop to the rectangle that would just enclose it. (From Griffiths et al, 1981, reproduced with permission from the Institute of Physics).

as the apexcardiogram. An example of the data generated by such a system is seen in Figure 2.1. Once the information is stored, further calculations can be performed on it. The position co-ordinates of one signal can be subtracted from those of another; for example, those of the endocardium can be subtracted from those of the septum to give ventricular dimension. Thereafter these co-ordinates can be differentiated to give their rates of change with time and finally combined with one another to provide more complex information, e.g. the apex – dimension loop. Briefly the apex-dimension loop is a way of detecting and displaying changes in left ventricular dimension during isovolumic contraction and relaxation. Such changes in left ventricular dimension during the isovolumic periods denote shape changes caused by inco-ordinate left ventricular function, commonly seen in ischaemic heart disease, or the volume changes when the ventricle should be remaining isovolumic, e.g. in aortic regurgitation (Venco et al, 1977). The normal loop is nearly rectangular with little change in dimension occurring during the two isovolumic periods (during the upstroke and downstroke of the apex-cardiogram). In addition, the peak rate of ventricular filling is known to be reduced in mitral stenosis (Hall et al, 1981) and in left ventricular hypertrophy (Gibson et al, 1979). Digitising also produces useful information about cavity size and wall thickness, although there is no reason to believe that these data will be more precise than information obtained from quicker, direct measurements made from original echo tracings. The clinical relevance of information concerning inco-ordinate left ventricular contraction, which can be obtained from the apex dimension loop (Doran et al, 1978), is less well established.

Reproducibility and Sources of Imprecision

These then are some of the measurements that can be made from M-Mode recordings, but little is known of their reproducibility and the errors associated with these measurements. It is often assumed that there is a 'true' value for every echo measurement, but this can only be an estimate. There are several factors relating to this value which may give rise to imprecision (Fig. 2.2). There are

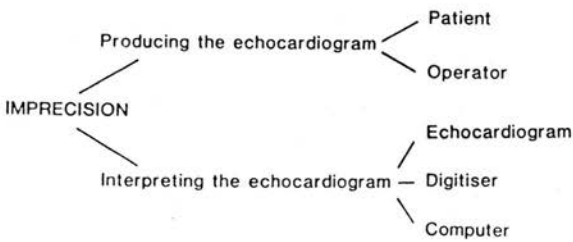


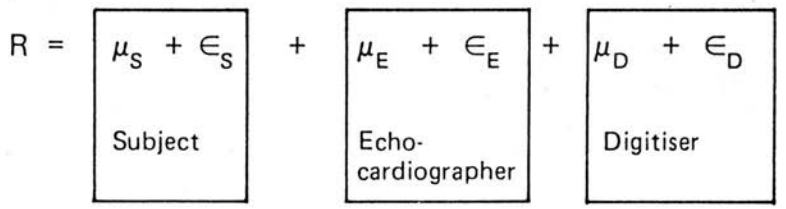
Fig. 2.2 Sources of imprecision in echocardiography.

errors associated with producing the echocardiogram: the patients may contribute errors or variability relating to his or her sex, shape, heart rate or associated diseases. Echocardiographer error may result from poor quality tracings in difficult subjects or the use of a non-standard transducer position (Popp et al, 1975), which may in turn relate to inexperience. Errors of

interpretation result from incorrect identification of intracardiac echoes, which again may reflect the quality of the echocardiogram and the experience of the digitiser. Finally, the computer system may contribute imprecision if the graphics tablet has poor resolution, the pen-cursor has a large surface area or if the digitiser fails to calibrate the digitising tablet correctly.

For every 'true' value there is a random error spread around the mean which can potentially be reduced by repetition. In addition, there can be potential by consistent error associated with an individual operator who consistently either over or under-estimates a measurement; this will not reduce with repetition.

This can further be explained by referring to Figure 2.3. In this formula, R is any single measurement reading, μ is the potential, consistent effect associated



μ – consistent effect associated with subject, echocardiographer, digitiser

$\in$ – random variability associated with subject, echocardiographer, digitiser

Fig. 2.3 Formula describing the relationship of the subject (S), echocardiographer (E) and digitiser (D) effects on echocardiographic measurements.

with the echo subject (S), echocardiographer (E), or digitiser (D), and $\in$ is the random effect associated with the subject, echocardiographer or digitiser' In the formula, μ_S is the 'true' value. In practice it is likely to be associated with a random subject effect $\in_S$ and further associated with random and possibly consistent echocardiographer and digitiser effects (μ_E, $\in_E$, μ_D, $\in_D$). Thus, for a given combination of subject, echocardiographer and digitiser, the mean of repeated readings will tend to reduce the random effect ($\in$) but not the consistent effect (μ). We have performed two studies to examine the reproducibility of echo measurements and their sources of variability. Both have used analysis of variance techniques to extract, where possible, the variability associated with performing M-Mode echocardiography and measurement of echocardiographic data. The important concept of variance is that if more than one aspect of a measurement contributes to variability, then the total variance is simply the sum of the variance of each aspect. Thus, analysis of variance is a statistical technique which allows the separation of a total variance into individual components, whose relative size indicate the proportion that they contribute to the total, and therefore the importance of that component as a source of imprecision.

The more frequently quoted value of standard deviation (SD) is simply the square root of each estimate of variance. It follows that although variance

components are additive, the standard deviations associated with each component are not; the overall SD will be smaller than the sum of the SDs of components.

ROYAL VICTORIA INFIRMARY STUDY

This study examined the relative size of the measurement inaccuracies when an echocardiogram of average quality was digitised. Neither the influence of different echocardiographers, nor the variability between sequential measurements on the same patient, were examined.

All echocardiograms performed in our department are graded according to quality; 70% are suitable for digitisation, 20% being of research quality. The 50% which are digitisable, but not of optimum quality, contain small discontinuities in the echo boundaries where subjective interpretation is required when digitising the trace (Fig. 2.4).

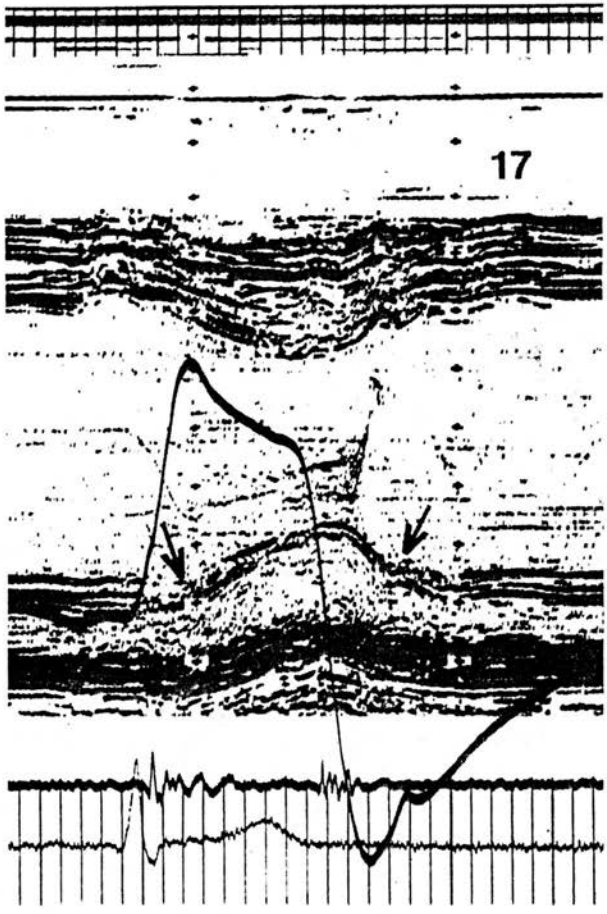


Fig. 2.4 An example of one of the 'average quality' echocardiograms used in Royal Victoria Infirmary Study. Areas of poor boundary definition are indicated by the arrows.

Methods

Five echocardiograms were selected which were of average digitisable quality, but not of 'research quality'. They had been obtained from normal young adult subjects, and contained simultaneously recorded apexcardiograms. From each echocardiogram four cycles which had the most distinct echocardiographic boundaries were cut out (Fig. 2.4). The total of 20 individual cycles were arranged in random order and then digitised by 4 digitisers. Digitisation was then repeated on a separate occasion to produce two replicate measurements (Fig. 2.5). A total of 160 observations were available for each of the measurements being studied.

The experimental design allows the data to be arranged in levels so that the different sources of variability can be examined (Fig. 2.5), and the results were subjected to analysis of variance (Snedecor & Cochran, 1967). Systolic and diastolic dimension for left ventricular cavity, left ventricular posterior wall, interventricular septum and apex-dimension loops were recorded and analysed.

A 'standardised echocardiogram' was prepared by taking a single representative cycle and drawing over the echo boundaries so that they were clearly defined (Fig. 2.6). This standardised echocardiogram was digitised 8 times by each of the 4 observers.

Results

The mean values for the measurements studied in the sample of five subjects all lie within normal ranges. The experimental results are expressed as components of the total variance amongst all the observations.

Left ventricular minor systolic dimension and wall thickness

For the left ventricular minor systolic dimension, the total variance of $0.14cm^2$ derived from the results on the five subjects is divided into a component due to variability 'between subjects' of $0.12cm^2$ (giving a standard deviation of $\pm$ 0.35cm) and variance components 'within subjects' due to cycle variability, $0.0044\ cm^2$ (SD $\pm$ 0.07 cm) (Figs. 2.7 and 2.8). The remaining variance was due to replication, that is the same cycle digitised by the same observer on a separate occasion, $0.0095cm^2$ (SD $\pm$ 0.1 cm). Adding the components gives a total 'within subject' variance of $0.019\ cm^2$ and a standard deviation of $\pm$ 0.14 cm.

Results for left ventricular minor dimension, posterior wall and interventricular septum are all similar, and presented diagramatically in Figure 2.8. The values of the 'between subjects', the between cycles and between digitiser 'within subject' variance are statistically significant. The proportion which the between cycle and between digitiser components contribute to the total 'within subject' variance is usually a smaller proportion than the more important variance component of replication. The 'between subject' variance component for these three measurements in systole and diastole was considerably larger than the 'within subject' variance (Fig. 2.8), except for diastolic septal thickness. The variance of the observation made on the standardised echocardiogram is much smaller than that of the 'within subject' variance of the study echocardiograms (Fig. 2.8, Table 2.1).

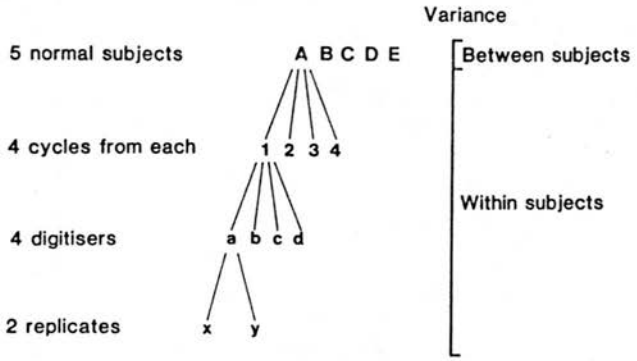


Fig. 2.5 The Royal Victoria Infirmary Study design, showing the levels of analysis used in the statistical method. 160 observations were made of each variable.

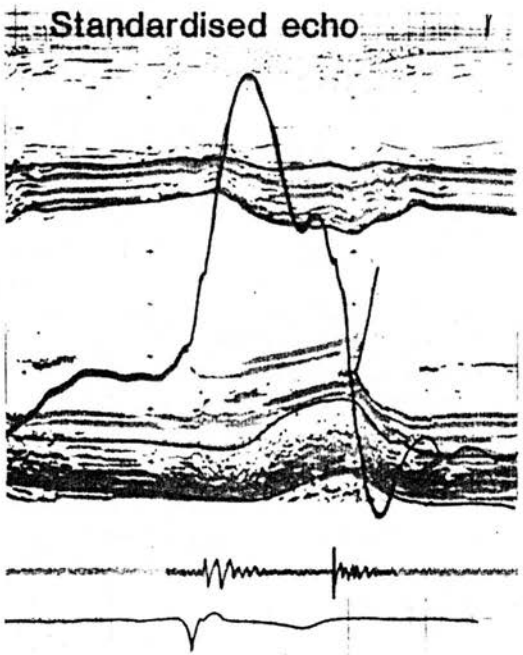


Fig. 2.6 The 'standardised' echocardiogram. The boundaries have been traced with black ink.

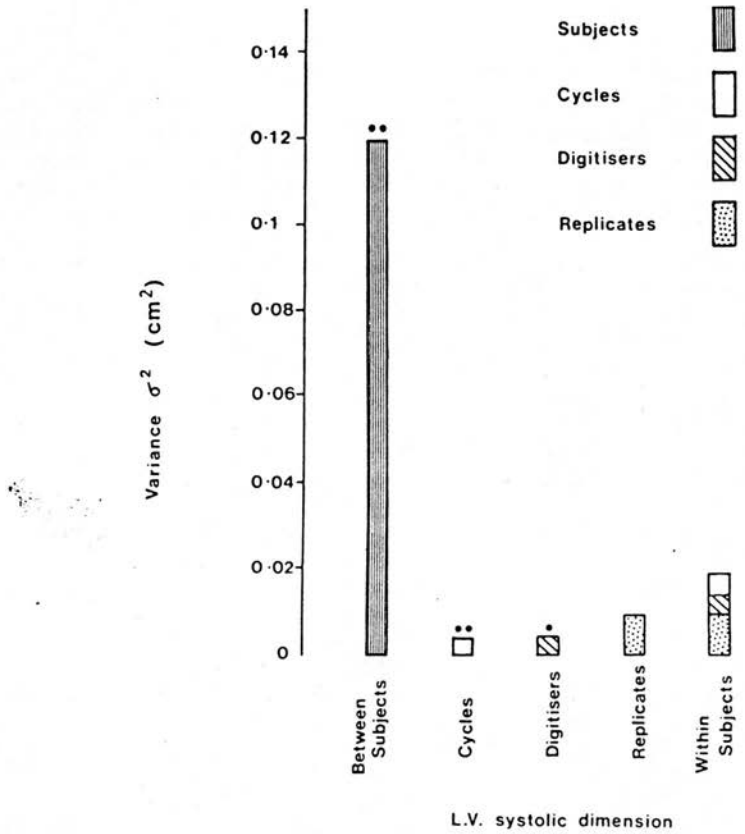


Fig. 2.7 Components of total variance (0.14cm^2) for LV systolic minor dimension.

Table 2.1 Standard deviations for observation on a normal echocardiogram and the 'standardised' echocardiogram, compared with those of Gibson and Brown 1973 (Royal Victoria Infirmary Study).

	Normal echocardiograms		Standardised echocardiograms		Gibson & Brown	
	systole	diastole	systole	diastole	systole	diastole
LV minor dimension	±0.14 cm	±0.16 cm	±0.04 cm	·0.07 cm	±0.17 cm	±0.16 cm
LV posterior wall	±0.15 cm	±0.12 cm	±0.04 cm	±0.03 cm		
Interventricular septum	±0.17 cm	±0.15 cm	±0.04 cm	±0.005 cm		
Peak rate of change	±1.3 cm/s	±3.3 cm/s	±0.8 cm/s	±1.5 cm/s		±1.5 cm/s

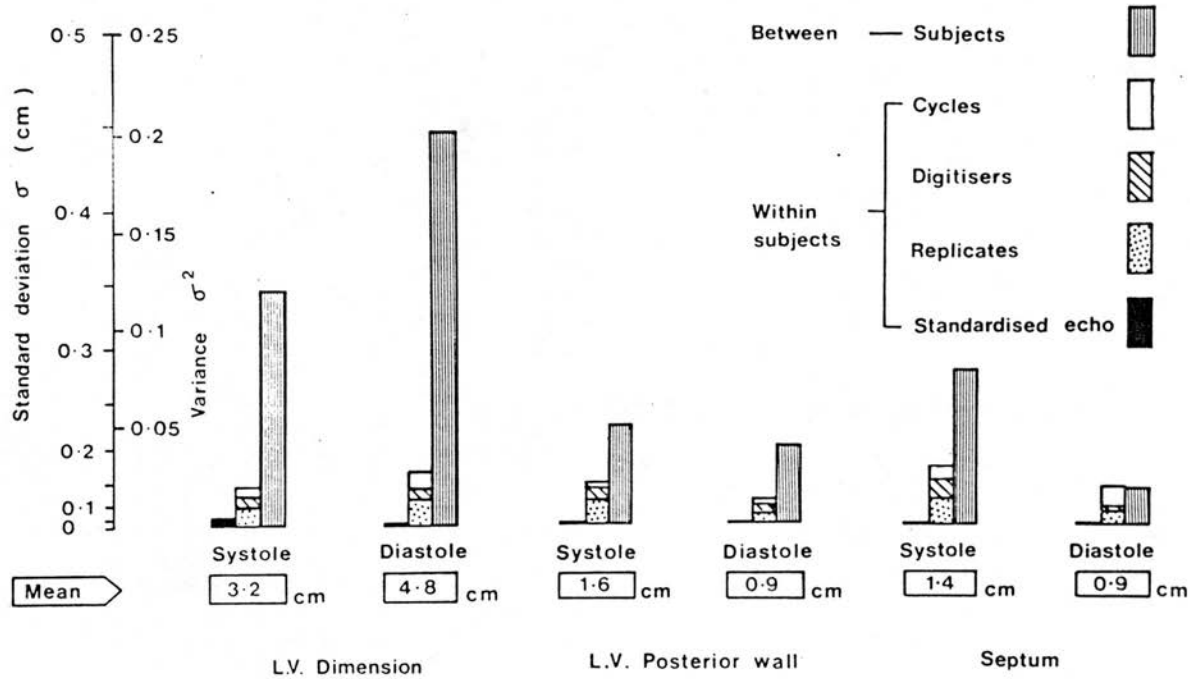


Fig. 2.8 'Between subject', and 'within subject' variance components for LV dimension and wall thickness, compared with variance of the standard echocardiogram.

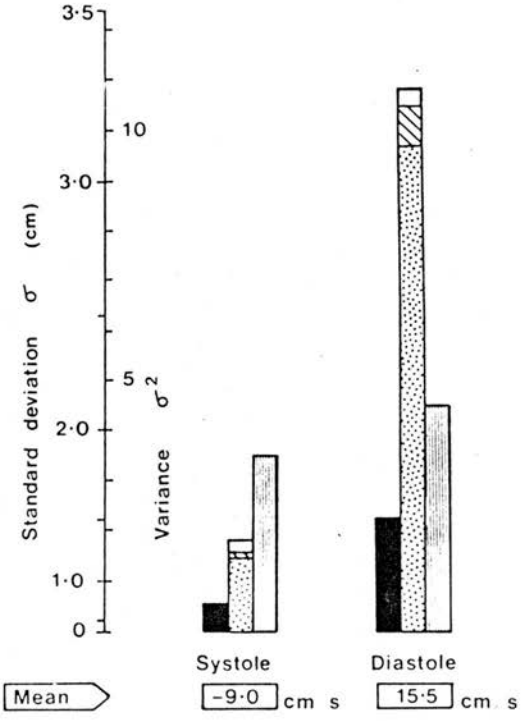


Fig. 2.9 'Between subject' and 'within subject' variance components for peak rate of change of LV dimension, compared to the standardised echocardiogram (key as for Fig. 2.8).

Peak rates of change of left ventricular dimension

The results for the derived variables of peak rate of change demonstrate much larger 'within subject' variance components compared to 'between subjects' variance (Fig. 2.9). In particular the replication component of variance is greatly increased so that for diastolic peak rate of change of dimension it is greater than the 'between subject' component. The 'within subject' standard deviation for peak rate of change of diastolic dimension is very large at ± 3.3 cm/s. The standardised echocardiogram also has a large variance component, considerably exceeding the value for both the cycle variation and the between digitiser variation in the results from the real echocardiograms.

Apex dimension loops

Quantitative data for the apex dimension loops is not presented, although a series of loops produced by two digitisers from the same echocardiogram are shown in figure 10 for qualitative analysis. A marked cycle to cycle variation is observed with small differences between the loops recorded on two separate occasions by the same digitiser, and on another occasion by a second digitiser.

FREEMAN HOSPITAL STUDY

This study examined the relative size of inaccuracies associated with repeated echocardiographic studies on a subject by different echocardiographers. The

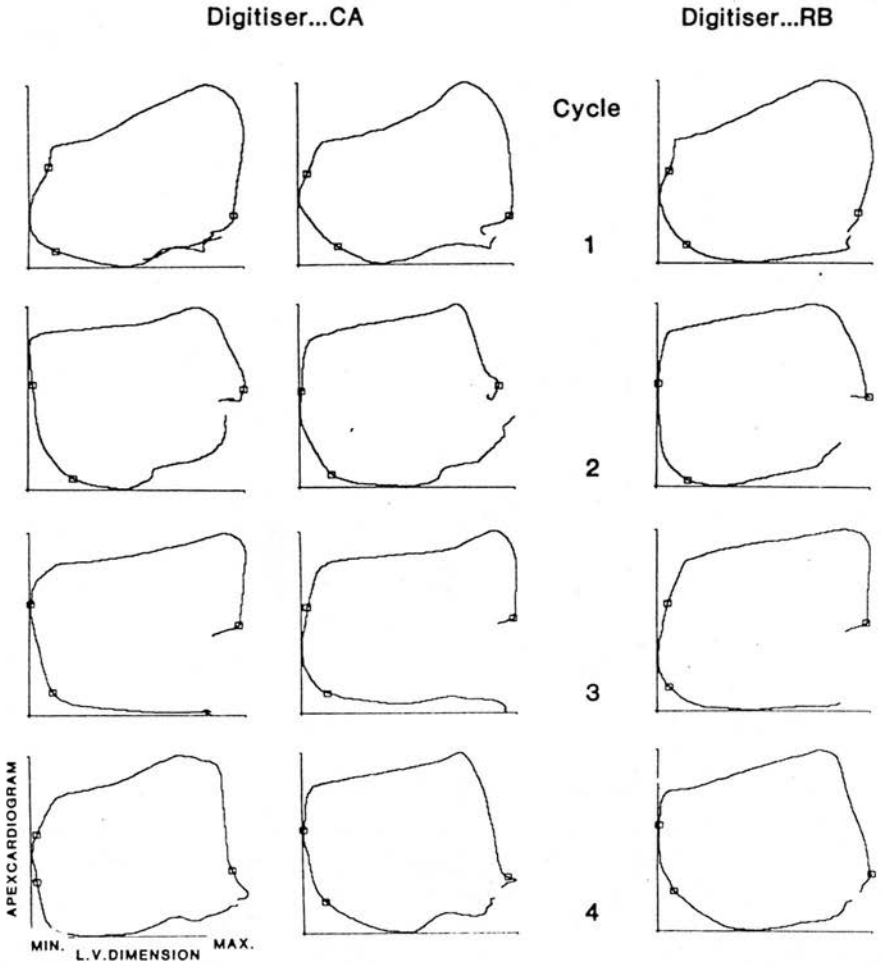


Fig. 2.10 Apex-dimension loops produced from four cycles of an echocardiogram from one of the normal subjects. Digitisation performed by one digitiser on two separate occasions, and again by another digitiser.

Freeman Hospital study looked at aspects of variability associated with obtaining the echocardiogram from the subject by different echocardiographers, and the data was analysed to separate consistent errors from random errors where possible.

Methods

In this study, 8 normal volunteers (4 of each sex), were studied by 4 experienced echocardiographers. The subjects were investigated in the left lateral position using conventional echo techniques. Echocardiograms were performed after a 10 minute rest period to obviate the effect of recent exercise. The order in which echocardiograms were performed was dictated by the requirements of a Latin Square (Fig. 2.11). All 4 echocardiograms on each subject were performed in a single session. The tracings were independently digitised by the operators who

STUDY DESIGN

		T1	T2	T3	T4
Males	P1	A	B	C	D
	P2	B	C	D	A
	P3	C	D	A	B
	P4	D	A	B	C
Females	P5	A	B	C	D
	P6	B	C	D	A
	P7	C	D	A	B
	P8	D	A	B	C

Echocardiographers, A B C D
Subjects P1–8
Time T1–4

Fig. 2.11 Echoes were performed in an order dictated by this Latin Square to separate out individual influences.

were unaware of the identity of the subject or the echocardiographer. All the tracings were digitised, irrespective of quality.

Results

In this study, the total variance is again divided into 'between subjects' and 'within subjects'. The 'within subject' variance was examined for contributions by replication and variation of adjacent cycles, both of which were assumed to be random. In addition, consistent differences between echocardiographers, digitisers and time or order of making the echocardiogram were sought, although the data available required such differences to be large before they would be statistically significant.

The variance components expressed as standard deviations are presented in Table 2.2 and graphically in Figures 2.12, 2.13 and 2.14 for measurements of posterior wall thickness, left ventricular minor dimension and peak rate of change of dimension. No significant adjacent cycle variation was observed.

Table 2.2 One standard deviation limits for measurements of normal subject echocardiograms (Freeman Study).

	Normal echocardiograms	
	systole	diastole
LV minor dimension	± 0.45cm	± 0.51cm
LV posterior wall	± 0.26cm	± 0.23cm
Peak rate of change	± 2.3cm/s	± 4.8cm/s

Posterior Wall Dimension

The total 'within subject' variance for the posterior wall thickness (Fig. 2.12) comprises a small digitiser component with a standard deviation of about ± 0.15 cm for a measurement on a single echocardiogram. The remainder of the variance is produced by different echocardiographers performing repeat investigations. The total 'within subject' variance for posterior wall dimension gives a standard deviation of ± 0.26 cm in systole and ± 0.23 cm in diastole. No consistent effects were identified for posterior wall dimensions.

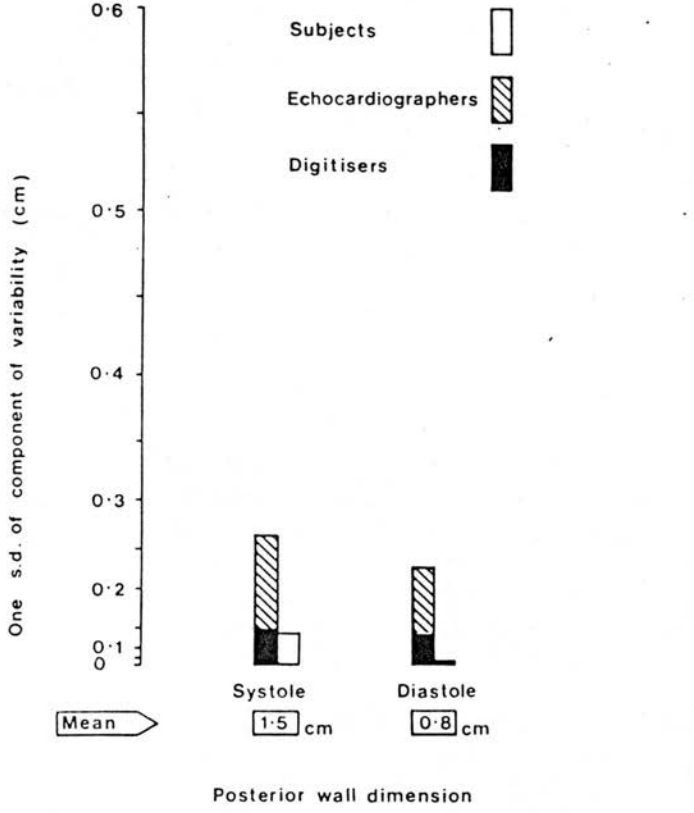


Fig. 2.12 Variance components for posterior LV wall dimension. (Unhatched histogram is subject scatter around the described mean).

DISCUSSION

Although there were important differences in the design of these two studies, there was considerable overlap which has allowed the major effects of variability to be compared.

The Royal Victoria Infirmary Study concentrated on the analysis of previously recorded echocardiograms. This design ensured that sufficient repeat measurements were made to allow an estimate to be made of all the aspects of variability studied.

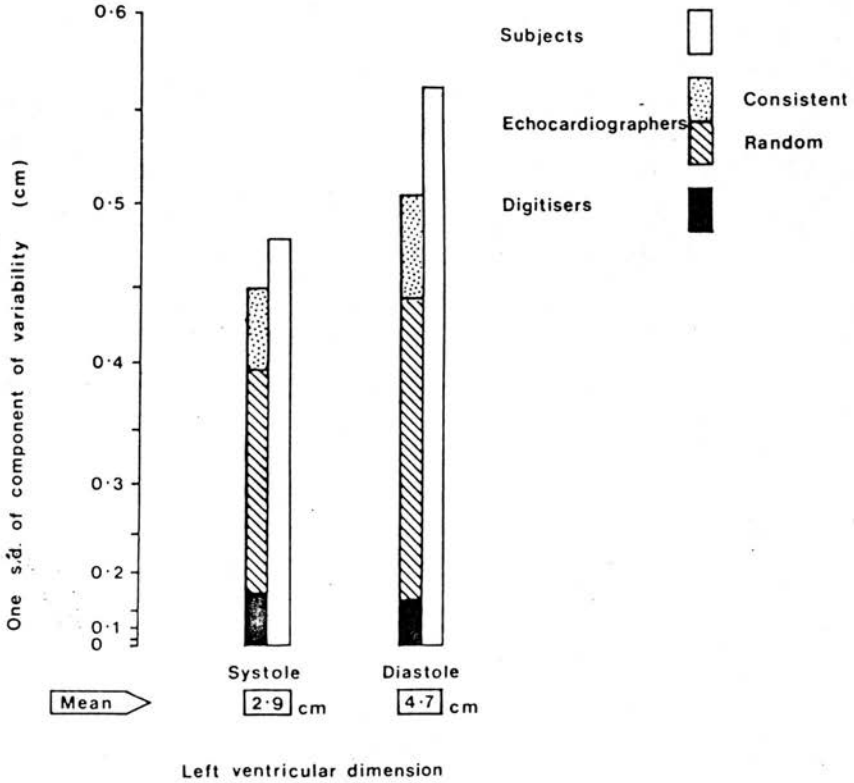


Fig. 2.13 Variance components for LV minor dimension. (Unhatched histogram is subject scatter around the described mean).

The Freeman Hospital Study additionally examined aspects of variability associated with performing the echocardiogram. Because of the widespread nature of the study, it was not possible to make sufficient repeat measurements to separate out all the components of variability. It did however, permit the components to be distinguished if there were sufficient differences. Thus, the two studies although different in design, were complementary.

These studies have demonstrated that M-Mode echocardiography and measurement of echocardiographic data can be associated with considerable errors. We have found that digitised errors of measurement of dimension are relatively small, but that digitised velocity measurement errors are large, especially during the filling phase. There is significant echocardiographer variability on both dimension and velocity measurements.

In the Royal Victoria Infirmary Study, in common with previously published studies we were able to demonstrate significant variability due to cycle to cycle variation and consistent differences amongst the digitisers (Bett & Dryburgh, 1981; Vignola et al, 1977; Belenkie, 1979). Although these variance components were statistically significant, their importance as sources of imprecision depends upon the size of their contribution to the total 'within subject' variance. For most

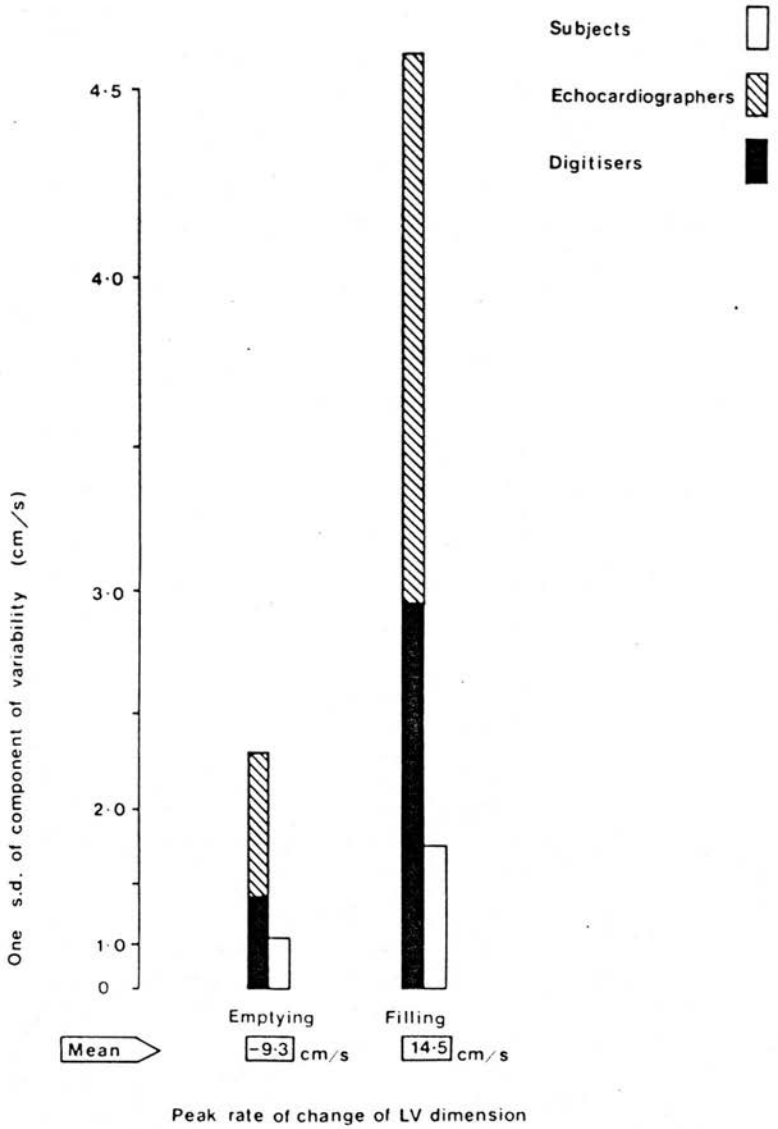


Fig. 2.14 Variance components for peak rate of LV dimension change. (Unhatched histogram is subject scatter around the described mean).

measurements studied the variance component for replication exceeded that for between cycles or between digitisers.

Since the replicate component is an important source of variance the accuracy of digitisation could be improved considerably if this element of the variance is reduced. Repeating the digitisation four times should reduce the standard deviation of the replication component by one half and therefore this would be a worthwhile manoeuvre.

Our method of analysis allows the total variance, or imprecision to be divided into its component parts. In order to differentiate one subject from another, and

therefore normal from abnormal, the 'within subject' standard deviation must be small compared to the normal range of values. The 'true value' has a 95% chance of being within ± 2 standard deviations of a single measurement'

For cavity size and wall thickness, the standard deviations are similar, approximately 0.15 cm (Table 2.1), so that a single measurement of cavity size gives a useful estimate of true value. As the absolute size of the wall thickness is much less than the cavity size, the standard deviation will be too large to allow differentiation of normal from abnormal and the precision of the measurement must be improved. This can be achieved by taking the mean of several digitised readings and so reducing the large replication variance component. The differing practical importance of this imprecision is illustrated by the relative sizes of the 'between subject' and 'within subject' variance components for cavity dimension and wall thickness (Fig. 2.8).

When the derived variable of peak rate of change is considered the diastolic 'within subject' variance component is found to exceed the 'between subject' component in our normal groups of subjects' The large replication variance component will be reduced to one quarter if four replicates are produced by repeated digitisation. This will reduce the standard deviation intervals by one half to ± 1.6 cm/s, almost identical to that obtained for a single measurement on a 'research quality' echocardiogram derived from the data of Gibson and Brown (1973), (Table 2.1).

The peak rate of change of left ventricular dimension in systole is less than in diastole (−9.0 and 15.5 cm/s) for our normal subjects. For both the normal subjects and the standardised echocardiogram the variance component for replication is considerably smaller for peak rate of change in systole compared to diastole. This suggests that the digitising technique may be more precise at lower rates of change, so that in the assessment of mitral stenosis where diastolic peak rate of change of left ventricular dimension is reduced (Hall et al, 1981), a more precise result may be obtained from digitisation than is expected from our data on normal subjects.

The results from the standardised echocardiogram should be the best that can be obtained with the digitising technique and equipment, as they are free of cycle to cycle variability and the echo boundaries are defined precisely. No significant variance was demonstrated between digitisers and the results are therefore equivalent to, and comparable with, the replication variance component of the normal echocardiograms (Table 2.1). Measurements of the standardised cavity size and wall thickness are much more precise than the normal echocardiograms, with very small variance components for the standardised trace, indicating as expected that improving boundary definition on the echocardiogram would result in greater measurement precision. The large variance for the standardised echo peak rate of change of left ventricular dimension, although smaller than those of the normal echocardiograms, indicate that the digitising technique itself is relatively imprecise in measuring rates of change. For diastolic peak rate of change of left ventricular dimension the standard deviation for the standardised echo was ± 1.5 cm/s: similar to that obtained by Gibson and Brown (1973) on 'research quality' echocardiograms (Table 2.1).

Apex dimension loops have been studied in order to aid understanding of left ventricular function but it is not yet clear whether they have any part to play in routine clinical investigations. The useful information in the loop is contained in the lateral up and down strokes which should not deviate from vertical by more than 15% of the total left ventricular dimension change. Our example (Fig 2.10) demonstrates considerable cycle to cycle variability. This is most likely to be due to variations in the shape of the apexcardiogram since cycle to cycle variation of the left ventricle has been shown to be small.

In the Freeman Hospital Study, the between digitiser component is in close agreement with the Royal Victoria Infirmary Study. It therefore confirms the large variance associated with digitiser measurement of wall thickness and peak rates of dimension change compared to the more acceptable result for left ventricular dimension. A large increase in variance is detected when repeated recordings are made by different echocardiographers. In addition, for measurement of left ventricular dimension, a proportion of this echocardiographic variance is shown to be due to consistent bias in technique amongst the different echocardiographers, the remainder of the variance being due to random variability introduced by repeat echocardiographic examination. No consistent effects are identified for posterior wall thickness, or peak rates of dimension change measurements.

The Apple microcomputer used in the studies is unlikely to generate much measurement imprecision as the graphics tablet has a resolution of less than 0.02 cm. The stylus of the digitiser commonly used on this type of computer does, however, have a rounded tip and is less precise than the cross wire cursor used by Gibson and Brown (1973). Another possible source of digitiser error is in calibration of the digitising tablet; depth and time marks have to be taken from the echo trace for every cycle which is digitised. The computer programme determines maximum and minimum values for diastolic and systolic wall thickness and cavity size. These may differ from conventional measurements where end-diastole is usually defined as the beginning of the QRS complex (Sahn et al, 1978).

CONCLUSIONS

These studies indicate the likely variability associated with performing and digitising echocardiograms. We have shown that echocardiographers can introduce large errors and it is likely that attention to standardisation of echocardiographic techniques would improve the reproducibility of echocardiographic data. In clinical practice, digitisation should only be performed on echocardiograms with adequate boundary definition. Furthermore, we have identified that an important source of variance is the replicate component. This is encouraging since, at least in theory, this element can be reduced by repeating digitisation (e.g. the standard deviation of the replication component can be halved by repeating digitising process four times).

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David Appleton

Precision of digitized M-mode echocardiograms for clinical practice

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KEY WORDS: Echocardiography–M-mode, precision, digitization, reproducibility.

Computer digitization of M-Mode echocardiograms has proved a valuable research tool in the study of left ventricular function. It can be applied in clinical practice if the reproducibility of the technique is known so that imprecision can be minimised. Five normal echocardiograms of average clinical quality were selected and four cycles from each echocardiogram were digitized on two occasions by four different digitizers using an 'Apple II' computer. The study design allowed the sources of imprecision to be identified by analysis of variance. Assessment of the clinically most useful digitized measurement, peak rate of change of diastolic left ventricular dimension (dD/dt), was imprecise with a large within-subject variance and a 95% confidence interval of $\pm 6{\cdot}5\ cm\ s^{-1}$*; too wide for clinical use. The replication component accounted for 90% of this variance, with cycle to cycle and between-digitizer variance components being much less important. This large replication component will be reduced simply by repeating digitization; for dD/dt repeating digitization four times would theoretically reduce the confidence interval to a useful value of* $\pm 3{\cdot}6\ cm\ s^{-1}$*. The 95% confidence interval for a single digitised measurement of diastolic left ventricular minor dimension was* $\pm 0{\cdot}32$ *cm, for diastolic posterior wall thickness* $\pm 0{\cdot}23$ *cm, and for the isovolumic relaxation time* $(A_2 - MVO) \pm 27$ *ms. Replication was again the largest component of variance so that the biggest improvement in precision will come from simply repeating digitization. Digitization on a cheap microcomputer system can provide clinically useful information provided reproducibility and sources of imprecision are known with clinical, as opposed to research, echocardiograms and conditions.*

Introduction

Computer digitization of M-mode echocardiographic boundaries, first described by Gibson and Brown in 1973[1], has become a useful research tool in the study of left ventricular function. Several systems have now been described, or are commercially available, where digitization is performed on a microcomputer graphics tablet[2]. These provide the possibility of digitized echocardiographic data being employed in routine clinical decision making, as in the assessment of patients with mitral stenosis[3].

Received for publication on 2 July 1984 and in revised form 20 August 1984.

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Echocardiography in clinical practice differs in many respects from research studies and less precise results may be expected. A variety of operators may be employed with individual variations in technique, and technically inferior echocardiograms may have to be accepted for clinical use. In addition research studies usually pool results from groups of patients to make comparisons between normal and abnormal populations, whereas a clinical decision is often influenced by a single echocardiographic observation. It is therefore essential to know the likely precision of an echocardiographic observation in normal clinical practice and it is not sufficient to rely on confidence intervals obtained in research studies. We have examined the relative size of the sources of imprecision which arise when a single echocardiographic tracing of average clinical quality is digitized, and estimated the 95% confidence limits for a single observation.

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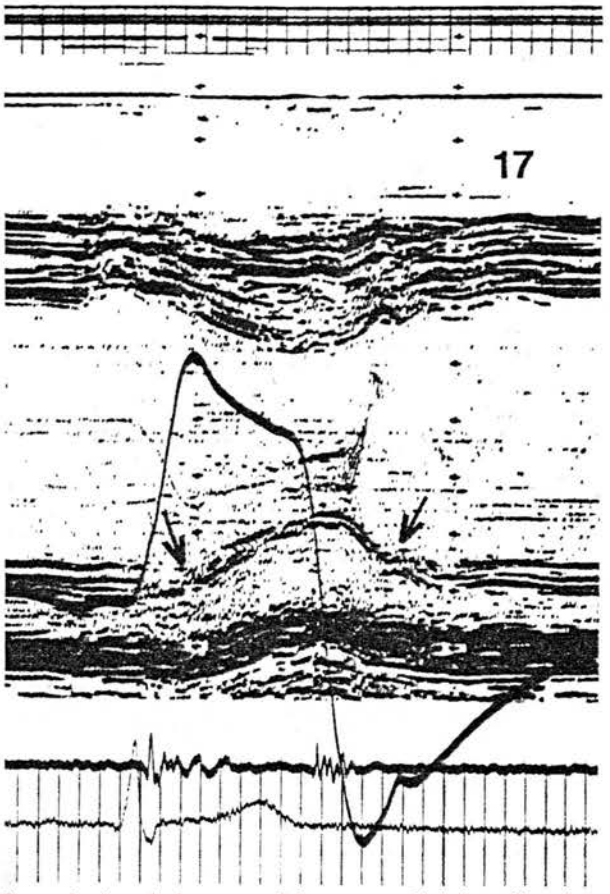

Figure 1 A cycle from one of the average clinical quality echocardiograms used in this study. Areas of poor boundary definition are indicated.

By investigating the source of our measurement error we are able to suggest ways of improving precision to produce clinically useful results. The influence of different echocardiographers, and the variability between sequential measurements on the same patient have been reported by previous authors and are not examined in this study[4–7].

Methods

Five echocardiograms of average clinical quality were selected, and four cardiac cycles showing the left ventricle at the level of the tip of the mitral valve leaflets were removed from each of the traces for analysis (Fig. 1). These echocardiograms had been recorded previously at a paper speed of 75 or 100 mm s^{-1} from normal young adult subjects and contain simultaneously recorded phonocardiograms. The 20 cycles were arranged in random order and then digitized by four individuals using an Apple microcomputer digitising system with software based on that of Gibson and Brown[1,2].

A pen plotter was used to record the leading edges of the echocardiographic boundaries onto a graphics tablet (resolution $<0{\cdot}02$ cm) and timing markers were plotted for the beginning of the aortic component of the second heart sound (A_2), and the point of initial opening movement of the mitral valve leaflet (MVO). Digitization was then repeated on a separate occasion to produce two replicate observations, giving a total of 160 observations for each measurement being studied.

The computer prints plots of cavity size and its rate of change, and posterior left ventricular wall thickness, and gives calculated maximum and minimum values of left ventricular minor dimension, wall thickness, and rate of change of dimension (Fig. 2). The isovolumic relaxation time

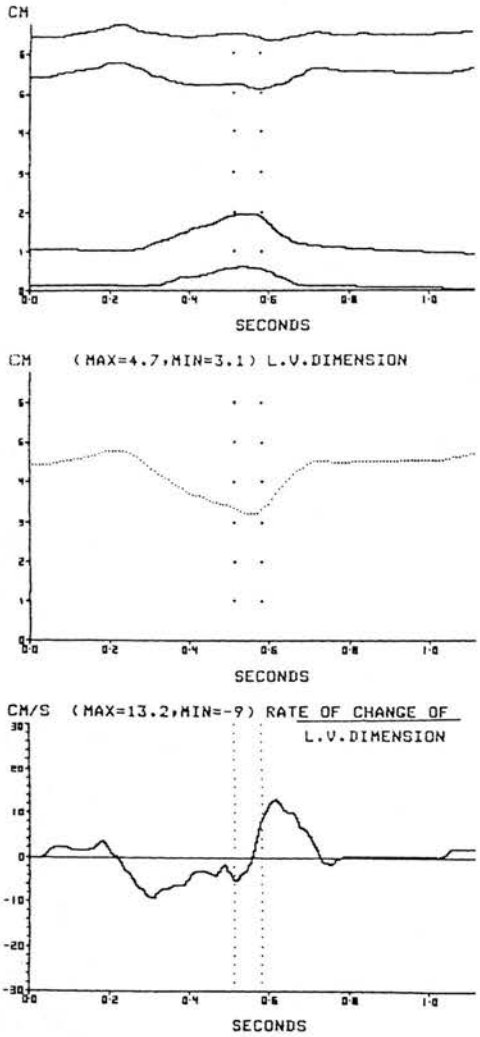

Figure 2 The digitized computer printout of left ventricular dimension and rate of change.

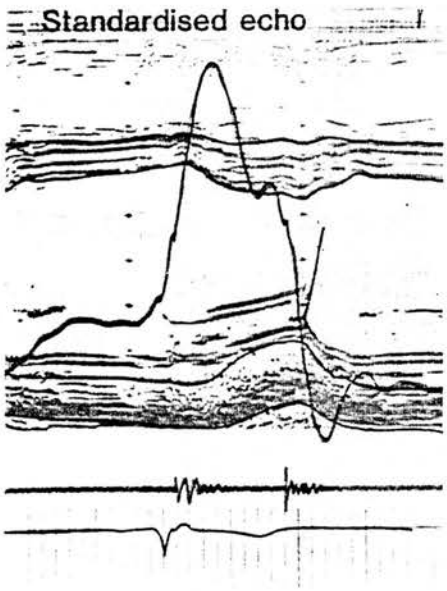

Figure 3 The standardized echocardiogram; boundaries are artificially defined.

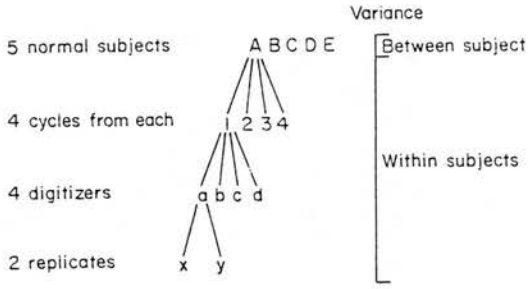

Figure 4 The study design.

(A_2 − MVO) was measured manually from these digitized computer traces and also directly from the original echocardiograms.

A standardized echocardiogram was prepared by drawing in the boundaries of a single representative cycle so that the investigator did not have to interpret the trace. (Fig. 3). This single standardized cycle was similarly digitized eight times by each individual.

Statistical methods

The experimental design allows the data to be arranged in a hierarchical manner so that an analysis of variance technique can be employed to obtain variance components for the different possible sources of imprecision[8]. The total amount of variability is divided into components due to differences between the subjects, and differences within the subjects (Fig. 4). The within-subject variance comprises a component due to differences between cycles of the echocardiogram, between different operators digitizing the same cycle, finally the replication component when the same investigator digitizes the same cycle on a separate occasion. These components of variance are additive, and from the total within-subject variance the standard deviation and thence 95% confidence interval for a single observation is obtained.

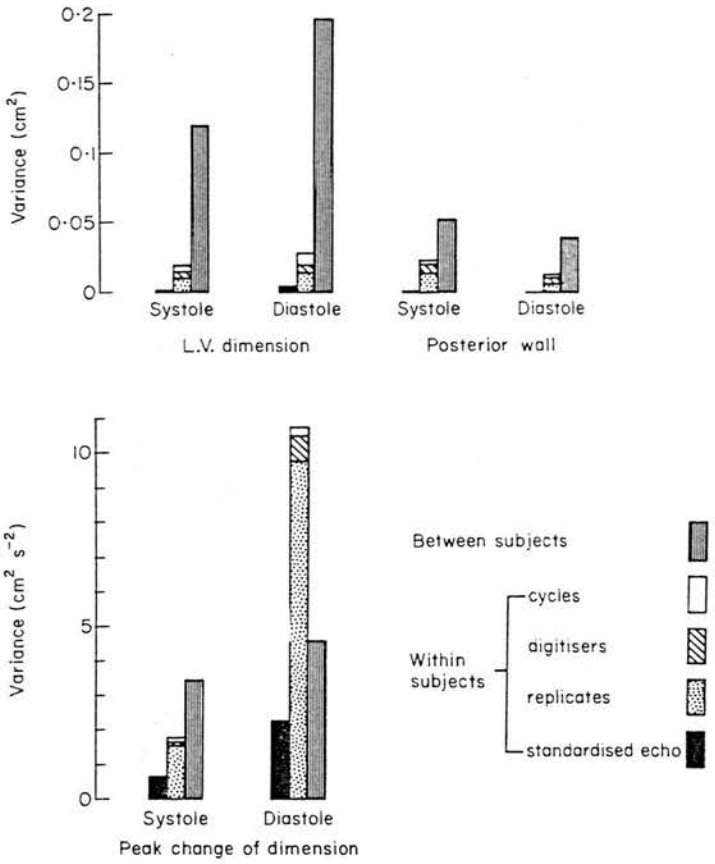

Figure 5 Variance components for left ventricular dimension, posterior wall thickness and peak rate of change of dimension.

Results

The mean values for the digitized echocardiographic measurements on each of the five subjects, and the standardized echocardiogram, were within our laboratory range of normality.

LEFT VENTRICULAR CAVITY DIMENSION AND WALL THICKNESS

The mean value for left ventricular dimension in this group of 5 normal echocardiograms was 3·2 cm in systole and 4·8 cm in diastole. Posterior wall thickness was 1·6 cm in systole and 0·9 cm in diastole. The total within-subject variance for left ventricular systolic dimension (0·019 cm^2) is much smaller than the variance between the subjects (0·12 cm^2) (Fig. 5). This total within-subject variance contains a component (24%) due to cycle to cycle variability (0·0044 cm^2), a similar sized component (26%) due to differences between digitizers (0·0048 cm^2) and a larger component (50%) of residual variance from replication of the observations (0·0095 cm^2). The total variance for measurements on the standardized echocardiogram is much smaller (0·002 cm^2), with the between-digitizer component accounting for only 5% of the variance, the remainder being due to replication. The 95% confidence interval for a single observation of left ventricular systolic dimension using any one cycle of the echocardiogram is $\pm 0{\cdot}27$ cm, compared with $\pm 0{\cdot}09$ cm for an observation on the standardized echocardiogram (Table 1).

Variance analysis of the results of left ventricular diastolic dimension and posterior wall thickness gives a similar pattern to that of left ventricular systolic dimension, with the between-subject variance components being larger than the within-subject components (Fig. 5). The replication component again accounts for approximately half

Table 1 95% confidence intervals for a single observation on a normal digitized echocardiogram and the 'standardized' echocardiogram

	Study echocardiograms		Standardized tracing	
	systole	diastole	systole	diastole
LV dimension	±0·27 cm	±0·32 cm	±0·09 cm	±0·13 cm
LV post. wall thickness	±0·29 cm	±0·23 cm	±0·08 cm	±0·05 cm
Peak rate of change of dimension	±2·6 cm s^{-1}	±6·5 cm s^{-1}	±1·6 cm s^{-1}	±3·1 cm s^{-1}
Isovolumic relaxation A_2 – MVO		±27 ms		±14 ms

of the within-subject variance, with equal sized components due to cycle variation and inter-digitizer variation. The 95% confidence interval for a measurement of left ventricular diastolic dimension is ±0·32 cm, and for posterior wall thickness ±0·29 cm in systole and ±0·23 cm in diastole. The confidence interval for the measurement of wall thickness on the standardized echocardiogram is much smaller at less than ±0·08 cm; no contribution to the variance was detected for differences between digitizers.

RATE OF CHANGE OF DIMENSION

The average peak rate of change of left ventricular minor dimension in systole was −9·0 cm s^{-1} for the group of five subjects, and +15·5 cm s^{-1} in diastole. Analysis of variance for the derived indices of rate of change reveal a much larger within-subject variance component in comparison to the between-subject variance component (Fig. 5). The proportion due to replication is greatly increased so that for diastolic peak rate of change of dimension it is larger than the between subject variance. The 95% confidence intervals are large at ±2·6 cm s^{-1} in systole and ±6·5 cm s^{-1} in diastole. The standardized echocardiogram also has a large variance component with 95% confidence intervals of ±1·6 cm s^{-1} in systole and ±3·1 cm s^{-1} in diastole (Table 1). No variance was detected due to differences between the digitizers on the standardized trace.

ISOVOLUMIC PERIOD TIMING INTERVALS

Measurement of the isovolumic timing interval (A_2 – MVO) made directly from the echocardiogram was a little less variable than that made from the digitized computer printout; 95% confidence intervals than that made from the digitized computer printout; 95% confidence intervals ±21 ms and ±27 ms, respectively (Fig. 6). Results for the standardized echocardiogram were more precise with a 95% confidence interval of ±14 ms on the digitized trace.

Discussion

In clinical practice M-mode echocardiograms are usually digitized to obtain measurements of instantaneous rates of change of left ventricular cavity dimension. Diastolic peak rate of change is known to be reduced in mitral stenosis[3] and left ventricular hypertrophy[9]. The digitizing process also produces useful information about cavity size and wall thickness, but there is little reason to believe that these results will be more precise or easier to obtain than those from quicker direct measurements on the original echocardiogram[7]. Our results for simple timing intervals similarly suggest that direct measurement from the echocardiogram may be more precise.

In our department we grade the technical quality of all M-mode echocardiograms recorded. Twenty per cent are of ideal quality suitable for research or teaching, 30% are of poor quality giving qualitative information only, the remaining intermediate 50% are of satisfactory quality for digitization but contain small areas where boundary definition is not ideal and subjective interpretation is required (Fig. 1). It is from this latter most typical group that our study echocardiograms were taken.

Our method of analysis allows the total amount of variance, or imprecision, to be divided into its component parts, and a 95% confidence interval to be derived from the within-subject variance, giving

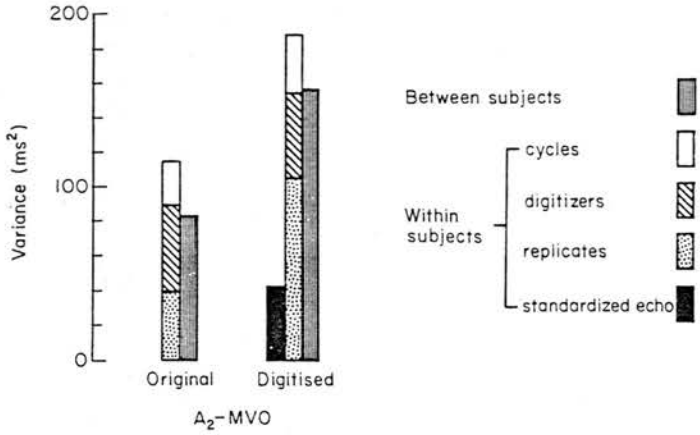

Figure 6 Variance components for the isovolumic period $A_2 - MVO$ measured directly from the echocardiogram ('original') and from the computer digitized printout ('digitized').

a guide to the likely discriminatory power of a single observation from a digitized echocardiogram. The confidence limit of $\pm 0{\cdot}32$ cm obtained for diastolic cavity size means that it is likely that a measurement can be classed as normal or abnormal as the confidence limit is small in comparison with the expected size and spread of normal values (normal $< 5{\cdot}8$ in our laboratory). The confidence interval of $\pm 0{\cdot}29$ cm will not be sufficiently narrow to make meaningful comparisons of wall thickness, where the range of expected values is small (normal $< 1{\cdot}2$ cm). For the most useful of the digitized values, diastolic peak rate of change of dimension, an observation with a confidence interval of $\pm 6{\cdot}5\ cm\ s^{-1}$ would not, over a considerable range of values, be able to differentiate between normal and abnormal left ventricular filling.

Analysis of variance indicates that the replication component is the largest of the within-subject components, and this will be reduced by repeating the measurements. It comprises 90% of the within-subject variance for the peak rate of change of dimension and the confidence interval will theoretically be reduced by approximately one half, to $\pm 3{\cdot}6\ cm\ s^{-1}$, if the measurements are repeated four times. In common with previous published studies we were able to demonstrate statistically significant variability due to cycle to cycle[10], and interobserver variation[4], but their importance as sources of imprecision is much less than that of the large replication component. Therefore to increase the precision of a digitized measurement it is most important to reduce this replicate component simply by repeating the measurement, although in practice it is prudent to reduce both replication and cycle-to-cycle variability at the same time by repeating the measurement on different cycles. Repeat digitization should be performed at different times so that a fresh unbiased interpretation of the echocardiogram is made on each occasion. The increase in precision which would be gained by digitization being repeated by another investigation is only small, and would probably not be justified in clinical practice.

The data on reproducibility for research quality echocardiograms in the original description of the digitizing technique by Gibson and Brown[1] give 95% confidence intervals of $\pm 0{\cdot}33$ cm for diastolic cavity size, and $\pm 3{\cdot}1\ cm\ s^{-1}$ for peak rate of increase in cavity dimension. This is similar to that which we would expect to obtain for our clinical quality echocardiograms by repeating digitization four times.

The within-subject variance is smaller on the slower systolic rate of change of dimension than for diastolic rate of change, suggesting that our digitizing technique may be more precise at slower rates of change, as found in most clinically important abnormalities.

The Apple microcomputer used in the study is unlikely to generate much measurment imprecision as the graphics tablet has a high resolution, although the pen plotter commonly used on this type of computer is less precise than the cross wire cursor used by more expensive systems. There will

however be an error associated with plotting depth and timing calibration points onto the computer for each cycle which is digitized. The computer programme determines maximum and minimum values for diastolic and systolic wall thickness and cavity size. These may differ from more conventional measurements where end-diastole is defined as the beginning of the QRS complex[11].

The results from the standardized echocardiogram can be regarded as the best that could be obtained with our digitizing technique and equipment, as they are free of cycle-to-cycle variability and the echo boundaries are clearly defined. Variances for measurements on the standardized trace are much smaller than for the real echocardiograms indicating, as expected, that improving boundary definition results in greater measurement precision. If boundaries cannot be defined on an echocardiogram the data produced will be misleading and another investigation technique should be employed.

Our results indicate the likely degree of imprecision to be expected when digitizing clinical quality echocardiograms on a simple microcomputer systems. However, other laboratories may differ in their definition of clinically acceptable echocardiographic quality, and should ideally perform reproducibility studies on their own material remembering that computerizing a measurement technique does not necessarily make it more precise. In the clinical setting observations may need to be repeated after an interval of time, and errors in obtaining the echocardiogram due to temporal variability and the influence of different echocardiographers are known to be at least as large as those from interpreting the echocardiogram[5,7]. We suggest that digitized results for peak rates of change of left ventricular dimension can be clinically useful provided digitization is repeated at least four times and the likely confidence interval for the digitizing system is known and taken into account when the data obtained is used to make clinical decisions.

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Chapter Two

Reproducibility in Cross-Sectional Echocardiography

I. Peart, A. Austin, D. Appleton and R.J.C. Hall

Introduction

Echocardiography is now playing an increasingly important role in the evaluation and management of patients with all forms of cardiac disease. In patients with ischaemic heart disease the two techniques of M-mode and cross-sectional echocardiography provide complementary information about left ventricular function. The advantage of M-mode echocardiography lies in the timing of cardiac events, identifying abnormal wall motion during the isovolumic periods of the cardiac cycle, i.e. during the upstroke and downstroke of the apexcardiogram (Doran *et al*, 1978; Upton and Gibson, 1978), as evidence of inco-ordinate left ventricular contraction – one of the hallmarks of coronary artery disease. Cross-sectional echocardiography, however, is a quick and convenient non-invasive way of obtaining direct visualisation of many left ventricular segments to assess regional left ventricular function, regional abnormalities of left ventricular contraction being another hallmark of coronary artery disease (Weyman *et al*, 1976; Kisslo *et al*, 1977; Heger *et al*, 1979).

This information about left ventricular function is of major importance and may help in the clinical management of individual patients. In patients with ischaemic heart disease it may help to distinguish between left ventricular aneurysm (Weyman *et al*, 1976; Hall, 1983) and globally poor left ventricular function as the cause of cardiac failure following myocardial infarction. In patients with valvular heart diseas it may provide sufficient diagnostic data t proceed with valve surgery without recourse t cardiac catheterisation (Hall *et al*, 1984) and als help in the timing of valve replacement. It als provides information that may have prognosti significance, e.g. following acute myocardia infarction (Horowitz and Morganroth, 1982 Jennings *et al*, 1984; Nishimura *et al*, 1984) an has been used in research to assess the effect o interventions such as exercise (Mitamura *et al* 1981; Morganroth *et al*, 1981) or surger (Rubenson *et al*, 1982) on left ventricula function in patients with ischaemic heart disease

As the two techniques of M-mode and cross sectional echocardiography have improved more and more clinical decisions are usin information derived from them. In many case the clinicians using such information do no understand its limitations in terms of accurac and reproducibility. Since the techniques ar being used in this way it is important that thei accuracy and reproducibility are established.

More studies have examined this problem i relation to M-mode echocardiography. We have some experience in that regard having already completed a detailed statistical study of the sources of error in digitised, normal, M-mode echocardiograms (Bullock *et al*, 1984b). This study showed that measurements taken from M mode echocardiograms can be associated with considerable errors (Table 2.1). Using echo cardiograms of average quality, but suitable fo digitisation, the digitised errors of simple

Table 2.1: Variability of measurements of simple and complex variables from digitised M mode echocardiograms. (Bullock *et al*. 1984)

	95% CONFIDENCE LIMITS	%
Simple		
LV dimensions	± 3mm	5%
LV wall thickness	± 1.5–3mm	12–25%
Complex		
LV dimension change: diastole	± 6cm/sec	40%
systole	± 3cm/sec	33%

variables such as measurement of LV dimensions are small, of the order of 5 per cent. However, when the distances measured are small, e.g. LV wall thickness, these errors, although of a similar magnitude, represent a larger proportion of the variable being measured (12–25 per cent). As for the more complex derived variables such as the rate of change of LV dimension, the errors are much larger, particularly during diastole (approximately 40 per cent) when dimension is changing quickly. The replicate component of variance was the largest component in all instances and could be reduced by half by repeating the digitisation on four occasions. When 'research' quality echocardiograms (i.e. those in which the echo boundaries are clearly visible throughout the cadiac cycle) were digitised the variance was reduced by half again.

Few studies have examined the problem in relation to cross-sectional echocardiography. Two well-designed studies by Moynihan *et al* (1981) and Parisi *et al* (1981) investigated computer assisted quantitation of both regional and global left ventricular function. In the first study (Moynihan *et al*, 1981) different approaches to the quantitation of regional left ventricular function were investigated to determine which yielded the best reproducibility between independent observers in a heterogeneous patient group and the least variability in a homogeneous group of normal volunteers. The data were then applied to a well-defined population of patients with coronary artery disease (Parisi *et al*, 1981) to evaluate the method's sensitivity to detect regional wall motion abnormalities and its specificity to identify normal left ventricular segments. The reproducibility of their technique was thus established.

In clinical practice, however, such computer aided analysis is often not available and cross-sectional echocardiograms are analysed by visual inspection. Several cross-sectional echocardiographic studies have analysed regional left ventricular function by visual interpretation of regional endocardial motion abnormalities (Heger *et al*, 1979; Mitamura *et al*, 1981; Morganroth *et al*, 1981; Rubenson *et al*, 1982; Nishimura *et al*, 1984). These studies have quoted good correlations between two or even three independent observers. When disagreement has occurred the problem has usually been resolved by consensus. In practice this probably means that the relative personalities and professional positions of the observers inevitably influence the results. None of the studies has specifically addressed the problem of reproducibility and none has tried to identify the sources of variability. In the past the problem of reproducibility has been assessed in relation to similar visual interpretation of left ventricular wall motion abnormalities from contrast cineangiograms. Studies of the problem in that situation showed that analysis based on simple visual inspection without measurement is inconsistent (Chaitman *et al*, 1975; Zir *et al*, 1976). A formal assessment of the reproducibility of visually interpreted cross-sectional echocardiograms is therefore needed.

STUDY DESIGN

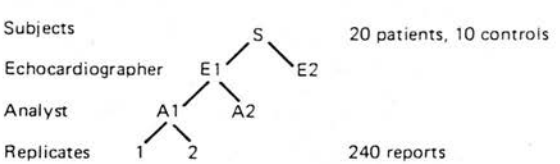

Figure 2.1 Study design showing the different levels at which variability may arise.

The present study was thus designed to evaluate the reproducibility of visual interpretation of left ventricular segmental wall motion from cross-sectional echocardiograms, and the method of data analysis was such as to allow the different sources of variability to be examined (Figure 2.1).

Reproducibility and Sources of Imprecision

The sources of variability in echocardiography arise at different levels, during both data acquisition and data analysis (Figure 2.2). Variability may arise during acquisition of the echocardiogram related to the patient's sex, shape or presence of other diseases such as chronic obstructive airways disease which makes adequate echo acquisition difficult. The echocardiographer can add to this by producing poor quality records or nonstandard views. Errors of interpretation may result from incorrect identification of echo boundaries which again may be related to the quality of the echocardiogram. In addition, individual analysers may vary in their interpretation of what constitutes normal or the different grades of abnormal wall motion. In an attempt to minimise this aspect of the problem the two analysers involved in the present study spent time together during the months prior to starting the formal study reporting echocardiograms from patients with ischaemic heart disease. These echocardiograms were assessed in the same way as in the study so that when the study commenced th two analysers would be working from a commo base when grading wall motion abnormalities. A further problem which may occur during analys relates to analyser bias towards reporting norma wall motion or certain grades of abnormal wa motion. Since any of these features of reportin may vary on repeated analysis of the sam echocardiogram, replication errors may als arise.

Until each of these areas is assessed the majo source or sources of variability cannot b identified and the reproducibility of th technique improved.

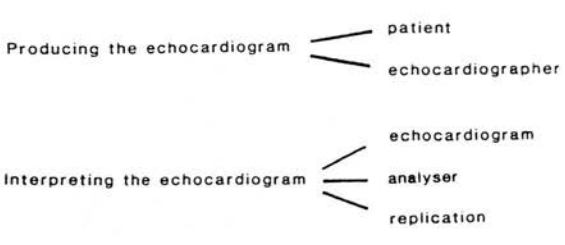

Figure 2.2 Sources of variability in echocardiography.

Patients and Methods

Patients

We studied 21 consecutive men undergoin coronary arteriography to assess angina. In on patient no analysable echoes could be obtaine and he was excluded from the analysis. Patient undergoing coronary arteriography for othe reasons, for example to assess prognosis fo lowing acute myocardial infarction, were no included in the study. Patients were not selecte for this study on the basis of being 'good ech subjects'. We included in the study group te patients who had had a previous myocardia infarction to ensure that regional wall motio abnormalities would be present in at least som of the subjects since without prior myocardia infarction or acute ischaemia, patients wit severely obstructed coronary arteries may hav normal left ventricular wall motion at res (Jacobs *et al*, 1973). A control population of te subjects of similar mean age and smoking habit was also studied.

Methods

Each subject had an echocardiogram performe by two experienced echocardiographers at th same session. Cross-sectional echocardiogram were performed using an ATL Mark mechanical sector scanner with a 3 mH

transducer. Each echocardiographer tried to obtain all of the same standard views (Figure 2.3) in each subject. The echocardiograms of both the controls and the patients were arranged randomly on videotape, the two analysers being blind to the randomisation. Some weeks later the echocardiograms were analysed by two analysers (Figure 2.1) and each analyser did this on two separate occasions. The completed analysis thus consisted of 240 reports.

In the past wall motion analysis has been investigated by subdividing the left ventricle in various ways (Weyman *et al*, 1976; Heger *et al*, 1979; Hecht *et al*, 1981; Visser *et al*, 1982) and using many different scoring systems. These methods have sometimes required the observer to choose by eye between five grades of abnormal wall motion in up to 20 or 30 different left ventricular segments. We chose to use a simple system, assessing left ventricular wall motion in five segments (Figure 2.3) and scoring this wall motion on a four point scale (Figure 2.4).

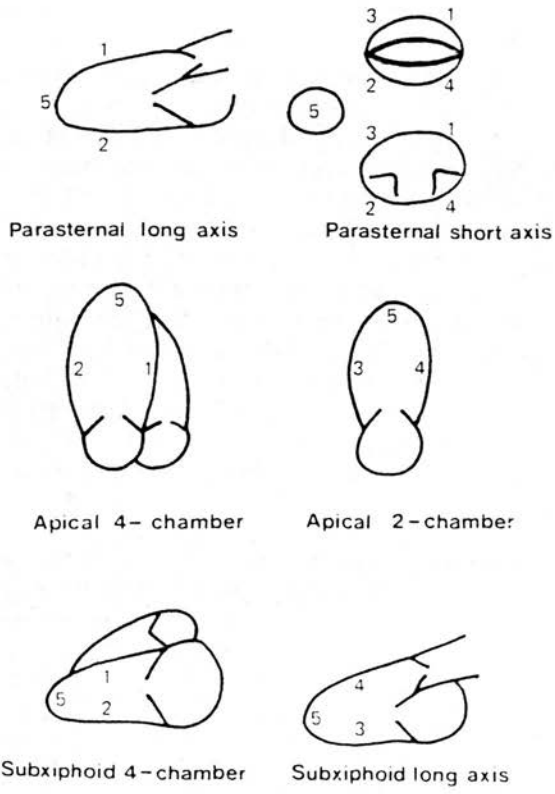

Figure 2.3 Standard echocardiographic views attempted in all subjects. The left ventricle is subdivided into five segments for wall motion analysis.

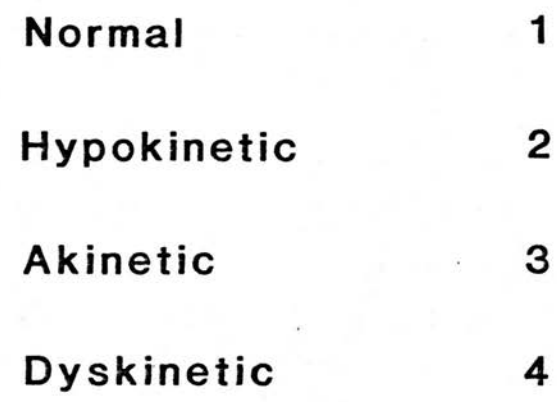

Figure 2.4 Wall motion score.

A segment was analysed only if more than 50 per cent of it was visible, if the endocardium was well visualised and if its motion could be assessed throughout the cardiac cycle. If any particular segment was not sufficiently well visualised to meet these criteria then the analysers had the option not to report.

In any individual echocardiogram a total of 24 possible reports could have been made on the five segments over all the different views (Figure 2.3).

Results

Segmental analysis

Table 2.2 shows the frequency with which each particular segment was reported by the two analysers. For the parasternal long axis and apical two and four chamber views reporting was almost universal except for the apical segment (No. 5) which was reported on only 20 per cent of occasions in the parasternal long axis view but on 90 per cent of occasions in the apical views. In the

Table 2.2: Frequency with which segments were reported by the two analysers in the different echocardiographic views

Parasternal long axis	97 - 100%
Apical 2CV & 4CV	97 - 100%
Parasternal short axis	73 - 84%
Subxiphoid 4CV & LA	20 - 40%

parasternal short axis views only 73–84 per cent of segments could be reported. Only 20–40 per cent of subxiphoid segments could be analysed.

Echocardiographers

There was no difference in the percentage of segments reported by the two analysers on the echocardiograms produced by the two echocardiographers in either the parasternal long axis, parasternal short axis or apical two and four chamber views (Table 2.3). However, more reports were made on the subxiphoid segments from echocardiographer 1 (30–50 per cent of segments were reported) than echocardiographer 2 (14–40 per cent of segments were reported).

Table 2.3: Contribution of echocardiographer to variability: frequency with which segments were reported by the two analysers in the different echocardiographic views from each echocardiographer

Parasternal long axis	No difference	
Apical 2CV & 4CV		
Parasternal short axis		
Subxiphoid 4CV & LA	Echo'er 1	30 - 50%
	Echo'er 2	14 - 40%

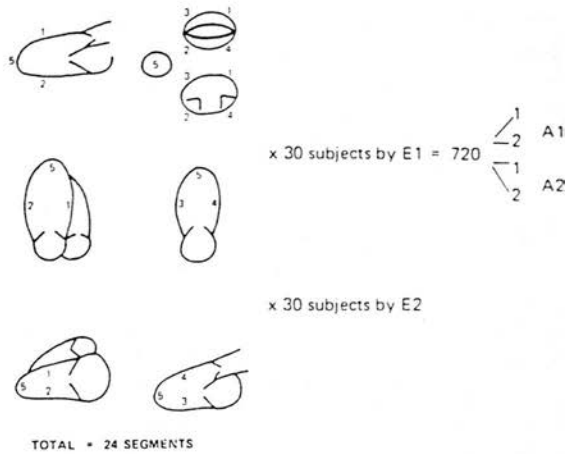

Figure 2.5 Segment groups compared to examine contribution of echocardiographer to variability.

Figure 2.5 further examines the contribution of the echocardiographer to the overall variability. Taking all possible views a total of 24 reports can be made on the five left ventricular segments in each of the 30 subjects studied by each echocardiographer – thus each echocardiographer could produce a possible 720 segments. Each segment could subsequently be reported four times by the two analysers, each on two occasions. Table 2.4 shows the number of times actual reports were made on every one of those four occasions. The difference between the echocardiographers was small (63 per cent: 55 per cent) and most of this difference was due to

Table 2.4: Frequency and consistency of reporting on the echocardiograms from each echocardiographer

	Echo'er 1	Echo'er 2
Possible groups	720	720
Analysed	457 (63%)	393 (55%)
4 times		
Agreed	289 (63%)	246 (63%)

the differing frequency with which echocardiographer 1 acquired echoes via the subxiphoid approach. The frequency with which all four reports were the same was the same for each echocardiographer (63 per cent).

Controls v patients

In the control group of ten subjects there are a possible 240 segments, and in the 20 patients 480 possible segments to be reported (Figure 2.6). Each of these segments could be produced by the two echocardiographers and analysed by the two analysers each on two occasions. Each segment could thus be reported eight times. Table 2.5 shows the number of times actual reports were made on every one of these occasions. This was achieved in only 50 per cent of segments in both the controls and the patients suggesting that there was no difference in the quality of the echocardiograms produced in the two groups. However, reporting was more reproducible in the control subjects. In the controls the graded assessment of wall motion was the same on every one of the possible eight occasions in 63 per cent of the segments reported; in the patients it was the same in only 45 per cent of the segments.

Table 2.5: Frequency and consistency of reporting in controls and patients

	Controls (10)	Patients (20)
Possible segments	240	480
Analysed (8 times)	123 (51%)	238 (50%)
Agreed (8 times)	78 (63%)	107 (45%)

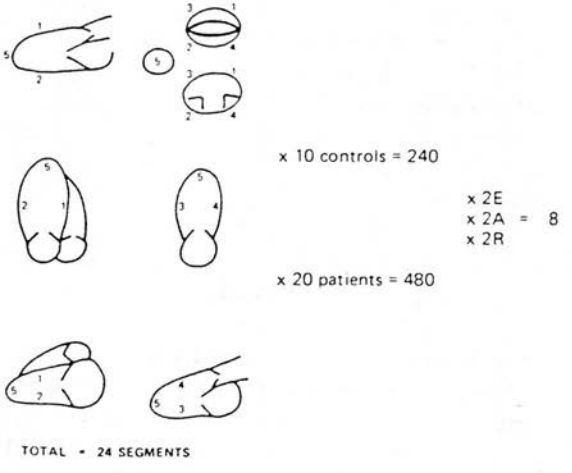

Figure 2.6 Segment groups compared to examine reporting patterns in controls and patients.

Analysers

Within analysers

Figures 2.7 and 2.8 examine the *within* analyser variability. The echocardiographers can produce a possible 720 segments from the 30 subjects. Each segment can then be reported four times by each of the analysers – two replicates from each echocardiographer. Table 2.6 shows the number of times actual reports were made on every one of those four occasions by each analyser. Analyser 1 tends to report more segments than Analyser 2 (68 per cent : 52 per cent), and Analyser 1 is more consistent in his reporting. He agrees with himself on every one of those four occasions 82 per cent of the time as compared to 59 per cent for Analyser 2.

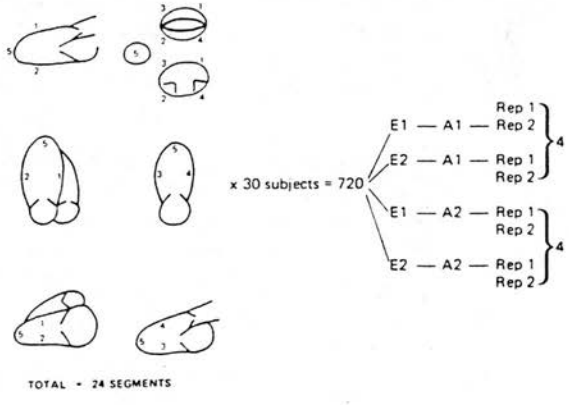

Figure 2.7 Segment groups compared to examine *within* analyser variability.

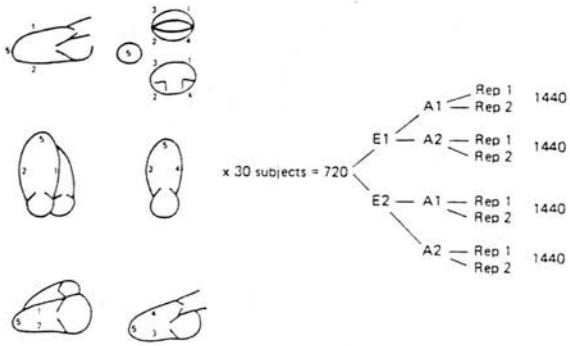

Figure 2.8 Segment groups compared to further examine *within* analyser variability.

Table 2.6: Frequency and consistency of reporting of each analyser

	Analyser 1	Analyser 2
Possible groups	720	720
Analysed	489 (68%)	375 (52%)
4 times		
Agreed	402 (82%)	222 (59%)

Within analyser variability can be further assessed by examining replicate analysis (Figure 2.8), i.e. the same segment from the same echocardiogram reported by the same analyser on two occasions (1,440 replicates). Again, Analyser 1 reports more replicates (74 per cent : 61 per cent) and is more consistent in his reporting (92 per cent : 80 per cent) than Analyser 2 (Table 2.7).

Table 2.7: Frequency and consistency of replicate analysis of the same segment by each analyser

	Analyser 1	Analyser 2
Possible replicates	1440	1440
Analysed	1062 (74%)	872 (61%)
twice		
Agreed	978 (92%)	695 (80%)

Between analysers

We examined the *between* analyser variability by comparing reports on the same segment from the same echocardiogram on the same occasion (i.e. comparing the first replicate report from analyser 1 with the first replicate report from analyser 2 etc) but by different analysers. There are 120 possible comparisons for each segment and in Table 2.8 we have outlined the results for four specific segments chosen because each showed different features of reporting. Parasternal long axis segment 1 was reported on almost every occasion and the *within* analyser agreement was good for both analysers. Parasternal long axis segment 2 was again reported on almost every occasion but the *within* analyser agreement was good for analyser 1 but not for analyser 2. Parasternal short axis segments 3 and 5 were chosen because the reporting frequency was low for both analysers.

The *between* analyser agreement varied from segment to segment, ranging from 63 per cent (parasternal long axis segment 2) to 89 per cent (parasternal short axis segments 3 and 5). Interestingly the *between* analyser agreement was highest in segments where the number of segments reported abnormal was low – 11/75 (15 per cent) in parasternal short axis segment 3 and 9/57 (16 per cent) in parasternal short axis segment 3. In parasternal long axis segment 2 where the majority of reports were abnormal (70/112 – 63 per cent) the *between* analyser agreement was only 63 per cent and in the majority of cases of disagreement Analyser 2 was grading higher (i.e. more abnormal) than Analyser 1.

Discussion

The cross-sectional echocardiogram is now well established as a means of noninvasively assessing left ventricular function (Reeder *et al*, 1982). In most centres this assessment is based on visual interpretation of left ventricular wall motion. As a guide to overall left ventricular function this type of analysis will probably suffice in most

Table 2.8: *Between* analyser variability

View	Segment analysed	Segments reported (120)	Reported abnormal	Agreement	Analyst I > II	Analyst II > I	
Parasternal Long axis	1	119	(32)	96 (81%)	14	9	(All 1 grade)
Parasternal Long axis	2	112	(70)	70 (63%)	6	36	(41 1 grade)
Parasternal Shot axis	5	57	(9)	51 (89%)	2	4	(All 1 grade)
Parasternal Short axis	3	75	(11)	67 (89%)	4	4	(7 1 grade)

instances. However, when more accurate assessment is needed such as when the effects of interventions or manoeuvres are to be evaluated, the analysis of left ventricular wall motion not only needs to be more precise but also reproducible. This problem of reproducibility has received much attention in relation to many aspects of M-mode echocardiography, and the sources of variability and the means by which this can be reduced have been carefully investigated (Bullock *et al*, 1984a). The same degree of investigation has not been applied to the problem in relation to cross-sectional echocardiography. The design and mode of analysis of the present study allowed us to examine the different levels at which variability may occur and also provided some answers as to why such variability arises.

The general applicability of the results depends in part on the frequency with which observations were made and, in common with previously published data from patients with ischaemic heart disease (Heger *et al*, 1979; Parisi *et al*, 1981), adequate visualisation of most parasternal long axis and apical two and four chamber segments was achieved (Table 2.2). In the parasternal short axis views only 73–84 per cent of possible segments were reported. Analysis of segments in these views, which involved assessment of four segments around the circumference of the left ventricle, proved to be difficult. In both the parasternal long axis and parasternal short axis views the apical segment (No. 5) was not sufficiently well visualised to allow reporting in the majority of subjects. Good quality cross-sectional echocardiograms were difficult to obtain via the subxiphoid approach, and this was reflected in only 30–40 per cent of segments being reported in these views.

The different levels at which variability can arise are listed in Figure 2.2. We examined the contribution of the patient by including a control group of subjects in whom the majority of left ventricular segments were expected to be normal. Only 50 per cent of segments were reported in all views in both the control subjects and the patients (Table 2.5). This suggests that the quality of the echocardiograms in the two groups was essentially the same and is probably explained by the fact that the control subjects chosen for this study were of a similar mean age to the patients and, in addition, some of the controls had been smokers. The consistency of reporting in the control subjects was better than in the patients (63 per cent : 45 per cent) and in the majority of cases the control subjects were reported as normal. However, there was one exception in whom both analysers agreed that left ventricular segmental wall motion was abnormal in a number of segments. Since the subject concerned had not undergone coronary arteriography these wall motion abnormalities

may be real. The patients in this study, therefore, contributed little variability.

To assess the contribution of the echocardiographer to variability and reproducibility is difficult. However, a crude assessment can be based on the frequency with which segments were reported on the echocardiograms of each echocardiographer, and also on the consistency of that reporting (Tables 2.3 and 2.4, Figure 2.5). It is likely that if a particular echocardiographer was producing either poorer quality echocardiograms or nonstandard views then this would appear as a lower number of reported segments. In this study the frequency (except for the subxiphoid views) and consistency of reporting was similar for both analysers on the echocardiograms produced by the two echocardiographers. Overall, therefore, it is likely that the echocardiographer contributed little variability.

The major finding in this study is that the analysers are the source of most of the variability. We have demonstrated large *within* and *between* analyser variability in the assessment of left ventricular segmental wall motion abnormalities. Tables 2.6 and 2.7 demonstrate the *within* analyser component of variability and shows both an increased frequency and consistency of reporting by analyser 1. Table 2.8 demonstrates the *between* analyser component of variability and shows that this varies in different segments.

Further assessment of the difference in frequency of reporting was not examined as part of the present study. This could only be achieved by joint re-analysis of the echocardiograms, the two analysers re-examining carefully those segments reported by one analyser and not the other.

The *within* analyser variability for analyser 1 was less than for analyser 2, analyser 1 being more consistent in his reporting. This may imply that his assessment of left ventricular segmental wall motion is really more reproducible although an element of reporting bias may also be involved. Analyser 1 may have a bias towards reporting normal as opposed to abnormal wall motion (i.e. reporting grade 1 rather than grades 2, 3, or 4) whilst analyser 2 is reporting mor widely across the grades. As the study grou comprises ten control subjects and ten patient with ischaemic heart disease but withou previous myocardial infarction, there is likely t be a predominance of normal segments. Bia towards reporting grade 1 would thus improv the likelihood of an analyser being consistent such a reporting pattern would also explain th improved consistency of reporting in the contro subjects as opposed to the patients (Table 2.5).

Table 2.9: Reporting patterns of each analyser

	Analyst 1	Analyst 2
Grade 1	1540	1202
Grade 2	418	462
Grade 3	206	198
Grade 4	0	14
Not analysed	716	1004

Table 2.9 shows the reporting pattern of the two analysers. Analyser 1 reports grade 1 more frequently than analyser 2 (1,540:1,202). However, the frequency of reporting grades 2 and 3 is essentially the same. Analyser 2 is thus not reporting more widely across the grades. Further analysis of the 338 occasions when a segment was reported as grade 1 by analyser 1 and not by analyser 2 shows that on 244 of these occasions analyser 2 felt that that particular segment was not sufficiently well visualised to make an accurate assessment of wall motion. These data combined with those presented in Tables 2.6 and 2.7 suggest that the more reproducible reporting by analyser 1 may include a genuine element of better reproducibility but there is also a suggestion of reporting bias in that analyser 1 is tending to report grade 1 (normal) wall motion when there is some doubt as to whether or not a segment is being visualised adequately.

Although accepting that one analyser is more consistent than the other in his reporting it is important to remember that consistency in

reporting does not necessarily reflect accuracy in assessment of left ventricular wall motion. Analysis can be consistently inaccurate and may be the case because of reporting bias in the forms previously discussed.

The assessment of *between* analyser variability demonstrates that, not surprisingly, agreement between the analysers was best in those segments (parasternal short axis segments 3 and 5) where the frequency with which abnormal wall motion was reported was lowest. In parasternal long axis segment 2 where the majority of reports were abnormal (70/112) the *between* analyser agreement was only 63 per cent. In the majority of cases of disagreement (36/42) analyser 2 was grading higher than analyser 1. These results would again suggest that analyser bias in reporting segmental wall motion is an extremely important variable.

Conclusions

This study has demonstrated that in patients with ischaemic heart disease segmental visualisation is variable, being best in the parasternal long axis and apical two and four chamber views, and worst in the subxiphoid views. The echocardiographer contributes little variability since the frequency and consistency of reporting were the same for the echocardiograms of each echocardiographer. The fact that reporting was more consistent in the control group shows that normal wall motion is easier to assess reproducibly than abnormal wall motion although some of this improved consistency may relate to patterns of reporting on the part of the analysers. The major source of variability in the assessment of left ventricular segmental wall motion by visual inspection lies with the analysers. The study has demonstrated large *within* and *between* analyser components of variability. This obviously has major implications if visual assessment of left ventricular wall motion is being used in longitudinal studies to assess the effects of surgery, drugs, etc. on left ventricular function.

Acknowledgement

We would like to thank the National Heart Research Fund who supported Dr Ian Peart while this work was being done.

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Breast-feeding and respiratory syncytial virus infection

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Summary and conclusions

The pattern of breast-feeding in 127 infants admitted to hospital with respiratory syncytial virus infection was compared with that in 503 age-matched controls. Thirty per cent of children with infection had been breast-fed compared with 49% of controls. The approximate relative risk of being admitted to hospital with respiratory syncytial virus infection if not breast-fed was 2·2. Several other factors were also considered, including an assessment of maternal care and home environment; the mother's age, marital state, and smoking habits; the number of siblings; and gestation. Adverse factors were all associated with an increased risk of admission with infection, but breast-feeding still appeared to provide

protection after controlling for these other factors in turn.

These findings provide further support for encouraging mothers to breast-feed their infants and should prompt further studies into the immune status of mothers and into the nature of the protective factors in their breast milk.

Introduction

Infants admitted to hospital with respiratory syncytial virus infection are less likely to have been breast-fed.[1 2] This may be because breast-feeding is protective. Nevertheless, several adverse social and environmental factors such as overcrowding, large families, and unemployment are associated with respiratory syncytial virus infection,[3] and these factors may also be linked with failure to breast-feed. It is therefore not certain whether breast-feeding itself is protective or is only a coincidental finding.

In an attempt to clarify this further we studied various epidemiological factors as well as breast-feeding in a group of children admitted to hospital with respiratory syncytial virus infection and in control children drawn from the general population.

Patients and methods

Children admitted to Tyneside hospitals with respiratory tract infections routinely have nasopharyngeal secretions and nose and throat swabs taken for viral diagnosis. The study included all infants aged 6 months and under in whom respiratory syncytial virus had been identified from the beginning of December 1977 to the end of March 1978. For each of the infected children four controls were selected from the birth register of the appropriate area health authority matched for date of birth. They were excluded only if they had left the area before the age of 7 months. Neonatal data were obtained from birth records.

During the spring and summer of 1978 the infected and control children were visited by their health visitors, who assessed the housing and maternal care of the baby and completed detailed questionnaires on family structure, environment, and breast-feeding. The health visitors' assessments were adapted from the study by Neligan and Prudham.[4] A four-point scale was used (1 = very good, 2 = satisfactory, 3 = unsatisfactory, 4 = very unsatisfactory) to score four aspects of housing (cleanliness, sanitation, furnishing, and structure), giving a total best score of 4 and a worst of 16. Five aspects of maternal care were scored (maternal attentiveness and affectionate interest, ability to seek appropriate help with illness, preparation of feeds, clothing and bedding, and cleanliness), giving a total best score of 5 and a worst of 20.

Results

Two hundred and fifty-one children were admitted to hospital with a respiratory syncytial virus infection during the winter of 1977-8. One hundred and twenty-seven (51%) of these were aged 6 months or under, and these children were included in the study. One hundred and seventeen had a lower respiratory tract infection and 10 had an upper respiratory tract infection. Five hundred and eight controls were selected. Questionnaires were returned for all index children and 503 controls, though a few were incomplete.

The pattern of breast-feeding is shown in table I. The odds ratio or approximate relative risk[5] of being in the virus-infected group if not breast-fed was 2·2 with 95% confidence limits of 1·4 and 3·5. The frequency of other possible risk factors in the virus group and in controls is shown in table II. Relative risk did not vary significantly with social class.

When the prevalence of breast-feeding was examined controlling separately for each of the factors in table II the relative risk of admission with respiratory syncytial virus infection if not breast-fed fell slightly but remained greater than 2·0 (Woolf's test[5]). For example, the relative risk in those with good maternal care was 2·0 and in those with poor maternal care 2·2; these two relative risks did not differ significantly and the overall relative risk was 2·0 (95% confidence limits 1·3 and 3·0). A logistic discriminant analysis of breast-feeding and seven other variables with the highest relative risks in table II suggested that maternal care, a mother's smoking, and the presence of another child in the same room at night were the most useful discriminators of risk. Breast-feeding lost its significance when we tried to allow for these three other variables at the same time but retained its significance when maternal care was excluded ($p < 0·05$) and when only maternal care and breast-feeding were considered ($p < 0·05$).

The duration of breast-feeding is shown in table I. The reduced risk of admission with infection was similar whether the child was still being breast-fed at the time of admission or not. The relative risk comparing those who had stopped breast-feeding and those who were breast-fed at the time of admission was 0·9.

TABLE I—*Patterns of breast-feeding in the virus-infected group and controls*

	Controls (n = 503)	Virus-infected group (n = 127)	Virus-infected group v controls	Virus-infected (tube-fed) group (n = 67)	Tube-fed infants v controls
No (%) breast-fed	239 (49)	38 (30)	$\chi^2 = 13·93$; $p < 0·001$	19 (28)	$\chi^2 = 9·52$; $p < 0·01$
No (%) breast-fed on admission or equivalent age ..	102 (20)	16 (13)	$\chi^2 = 4·25$; $p < 0·05$	10 (15)	$\chi^2 = 1·21$; NS
Mean (± SD) duration of breast-feeding (weeks) ..	11·9 ± 12·5	9·4 ± 11·8		10·4 ± 12·4	

Sixty-seven babies admitted with infection were sufficiently ill to need at least some tube feeds or intravenous fluids to maintain a normal fluid intake, and they were looked at separately. The proportions with adverse factors in the tube-fed group and in the whole group with infection were similar (table II). Nineteen of the tube-fed group had been breast-fed and their duration of breast-feeding was similar to that in the more mildly affected infants. A comparison of only tube-fed babies with controls showed an increased relative risk of admission with respiratory syncytial virus infection of 2·3 in infants who were not breast-fed.

TABLE II—*Possible risk factors in infants with respiratory syncytial virus bronchiolitis compared with controls*

	No (%) of controls (n = 503)	No (%) of virus-infected group (n = 127)	Relative risk	No (%) of virus-infected (tube-fed) group (n = 67)	Relative risk
Mother's care poor (score > 10) ..	40 (8)	32 (25)	4·1***	16 (24)	3·8**
Single mother	20 (4)	16 (13)	3·7***	11 (16)	5·0**
Another child sleeping with baby	40 (8)	28 (22)	3·2***	15 (23)	3·3**
Gestation < 36 weeks ..	20 (4)	13 (10)	2·7**	11 (16)	4·5**
Housing poor (score > 8)	86 (17)	44 (35)	2·7***	23 (34)	2·5**
Mother smokes	206 (41)	80 (63)	2·5***	46 (68)	3·1**
Father off work	75 (15)	38 (30)	2·4***	18 (27)	2·2*
Mother < 20 years ..	55 (11)	25 (20)	2·0*	13 (19)	2·0
One or more siblings ..	252 (50)	83 (65)	1·9*	42 (63)	1·7
Birth weight < 10th percentile	65 (13)	27 (21)	1·7*	17 (25)	2·2*

p value by χ^2 test compared with controls: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Discussion

Breast-feeding appears to halve the risk of admission to hospital with respiratory syncytial virus infection. Many adverse social factors are also associated with infection and all these factors tend to be associated with each other. The variation in risk with social class was too small to reach significance in the sample under study (relative risk 1·4). In contrast, the strong association with maternal care (which mirrors the earlier findings of Neligan and Prudham with regard to growth, non-verbal intelligence quotient, and school behaviour in later childhood[4]) shows that there are limitations to the epidemiological value of conventional social-class labels. In assessing maternal care the health visitors may instinctively have combined many of the factors that are important in influencing the development of respiratory syncytial virus infection. In addition, knowledge of the previous hospital admission might have influenced what was inevitably a subjective form of assessment. This would explain why maternal care was such a powerful discriminator.

Breast-feeding would also be one of the factors likely to influence the assessment of maternal care, and the consequent close association between the two explains why inclusion of maternal care in a logistic discriminant analysis causes breast-feeding to lose its significance when several factors are considered together. Of the mothers who breast-fed, 94% were assessed as providing good maternal care; this compared with 81% of mothers who did not breast-feed ($p < 0.001$). Even so, when only maternal care and breast feeding were considered breast-feeding retained its significance. We therefore consider that breast-feeding may be an independent factor influencing infection with respiratory syncytial virus.

One possible explanation for the reduced number of breast-fed babies in the group admitted to hospital might have been a greater reluctance to admit a baby who was being breast-fed if its mother was unable to come in as well. But the analysis of the more severely affected infants, who required tube-feeding and were unlikely to have been kept at home however good the circumstances, showed results similar to those for the whole group, suggesting that home circumstances were not influencing admission to any great extent.

Breast-feeding certainly does not give complete protection. Work done by Toms *et al*[6] has shown that breast milk varies in its antirespiratory syncytial virus activity; only four of 16 mothers tested had high levels of IgA specific for respiratory syncytial virus and five of 17 had lymphocytes sensitised to respiratory syncytial virus in their milk. This probably relates to recent exposure of the mother to the virus.

It is interesting that infants do not need to be breast-fed at the time of exposure to infection to be protected; there appears to be a lasting effect. This may be due to the fact that lymphocytes in the colostrum or milk that are sensitised to respiratory syncytial virus colonise the infant's nasopharynx, or to stimulation of the infant's own immune response by transfer of sensitised T cells or of antigen on macrophages. Recent work has suggested that the IgE response in the respiratory tract may be important in the pathogenesis of bronchiolitis,[7] and breast milk in rats has been shown to suppress the IgE response.[8]

This demonstration that breast-feeding probably influences respiratory syncytial virus infection in infants suggests three possible approaches to the problem. Firstly, it gives another reason for encouraging mothers to breast-feed their babies. Secondly, it has prompted us to look further at the immune status of mothers and how this is reflected in their milk and in their infants: boosting mothers' immunity might increase the protection they give to their babies. Thirdly, it provides a stimulus to the investigation of the nature of the protective factors in milk, helping to increase our understanding of immunological defences against respiratory syncytial virus infection.

We acknowledge the invaluable help given by health visitors in the Gateshead, Newcastle upon Tyne, North Tyneside, and Northumberland Area Health Authorities. This study would not have been possible without their help.

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Short Communication

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PROPRANOLOL AND GIVING UP SMOKING

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Summary

A placebo-controlled double-blind clinical trial was conducted to determine whether propranolol helps cigarette smokers to stop smoking; 73 subjects entered the trial but at the end of eight weeks only six had stopped smoking, three in the propranolol and three in the placebo group. There was no evidence of any helpful effect of propranolol in subjects trying to stop smoking.

Introduction

There is to date no effective pharmacological aid for cigarette smokers who wish to stop smoking (Royal College of Physicians 1977), except perhaps for the use of nicotine chewing gum (Russell et al. 1976), during which the possible harmful effects of nicotine continue. Propranolol is effective in relieving the somatic symptoms of anxiety (Leading Article 1976) and may help in the withdrawal of alcohol (Carlsson 1976), but earlier reports that propranolol was of help in opiate withdrawal have not been confirmed in double-blind studies (Resnick et al. 1976). We have conducted a placebo-controlled double-blind clinical trial to determine whether propranolol helps cigarette smokers to stop smoking.

Subjects and Methods

General practitioners were invited to refer cigarette smokers who wished to stop smoking and suitable subjects gave their informed consent. Subjects who were, or might be, pregnant and others in whom propranolol would be contraindicated were excluded (Visit 1). The subjects kept diary cards of daily cigarette consumption for 10 weeks. Treatment with propranolol 40 mg three times a day or matching placebo tablets administered double-blind was commenced at the beginning of the second week, but subjects were advised not to try to give up smoking at this stage because we wished to monitor any effect of the drug on pulse and blood pressure before cigarette consumption ceased. At the beginning of the third week (Visit 2) they were advised to try to stop smoking and were seen again at the end of the sixth week (Visit 3) and of the tenth week (Visit 4).

Seventy-three subjects entered the trial, 49 at the West Chest Clinic, Newcastle Upon Tyne, and 24 at St Thomas's Hospital, London. Details of the subjects in the propranolol and placebo groups are shown in Table I. Subjects completing each visit are shown in Table I. There was a high drop-out rate. The difference in attendance patterns between the propranolol and placebo groups was not significant ($\chi^2 = 3.94$ with 3 d.f.).

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Table I. Details of subjects and number of visits completed

	Propranolol	*Placebo*	*Total*
No. of subjects	35	38	73
Male	20	20	40
Female	15	18	33
Age (median and range)	39 (19–59)	36 (21–64)	
Cigarettes per day at entry (median and range)	31 (6–60)	28 (15–60)	
No. of visits completed:			
1	35	38	73
2	31	27	58
3	17	17	34
4	10	9	19

Results

Of the 19 subjects at Visit 4, six had stopped smoking, three on propranolol and three on placebo. Another 10 of the 34 subjects who completed at least three visits had reduced their cigarette consumption by at least one half (for at least two weeks) by the time they left the trial. Five had received propranolol and five placebo.

Discussion

This study failed to demonstrate any helpful effect of propranolol in subjects trying to stop smoking.

The drop-out rate was high and the number stopping in both propranolol and placebo groups was disappointingly small. Many of the subjects had already tried other methods to stop smoking and failed. Despite this the lack of difference between the propranolol and placebo groups suggests that any effect of propranolol which may have been missed because of the small numbers would be small and not clinically useful.

Acknowledgements

We thank the Newcastle Chronic Bronchitis Survey (Dr A. G. Ogilvie, Dr C. Strang, Professor D. Newell, and Dr P. O. Leggat) for financial support; Imperial Chemical Industries Ltd, Pharmaceutical Division, for supplying the propranolol and placebo tablets; Miss E. Hutchinson and Miss M. Edwards for help in running the trial; and Miss Suzanne Cox for typing.

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Disturbances in Lactate Metabolism in Patients With Liver Damage Due to Paracetamol Overdose

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Six patients with liver damage following paracetamol overdose, one patient with viral hepatitis and six control subjects were infused with sodium L(+) lactate. In controls the results were analysed using a single compartment model while in paracetamol patients a two compartment system was used to derive the fractional rate removal constant and lactate distribution volume. Forearm arterio-venous differences of lactate were also determined in order to assess the role of voluntary muscle in removal of a lactate load. In paracetamol patients the fractional rate removal constant was decreased to less than half the control value ($P < 0.001$) while total distribution volume was similar in the two groups. Fasting lactate concentrations were significantly increased in paracetamol patients due to diminished lactate removal since the endogenous production rate of lactate was not significantly different from controls. A greater proportion of the lactate load was removed in voluntary muscle in paracetamol patients (39%) than controls (17%). Since the balance of lactate removal occurs principally in the liver, the decrease in the fractional rate removal constant in patients following paracetamol overdose indicates a severe derangement of hepatic lactate metabolism with a compensatory increase in lactate metabolism in voluntary muscle.

A DISTURBANCE in lactate metabolism is well recognised in patients with liver damage, and underlying liver disease may be important in the pathogenesis of lactic acidosis. Fasting blood lactate concentrations were significantly increased in patients with viral hepatitis[1] and paracetamol induced liver damage,[2] and in patients with fulminant hepatic failure a three fold elevation in blood lactate was thought to be due to a combination of pH stimulated glycolysis and impaired gluconeogenesis.[3] Paracetamol (acetaminophen) taken with suicidal intent, frequently leads to a form of hepatic damage which may range from mild to severe hepatic necrosis.[4] When severe, hepatic coma may supervene and this is frequently fatal. However, many patients develop quite marked liver damage which is rapidly and completely reversible and in these there may be no evidence of hepatic encephalopathy. In order to elucidate the mechanism of the hyperlactataemia associated with liver damage, a group of such patients together with one patient with viral hepatitis were infused with sodium L (+) lactate and a number of indices of lactate metabolism determined. Arterio-venous lactate differences in the forearm were also measured in order to assess the relative role of liver and voluntary muscle in removal of a lactate load. The results indicate that the elevated fasting concentrations found in patients with paracetamol induced liver damage of moderate severity are due to a decrease in lactate removal by the liver while a compensatory increase in lactate removal in voluntary muscle was also observed.

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Received for publication July 23, 1979.

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0026-0495/81/3007-0002$01.00/0

MATERIALS AND METHODS

Six patients suffering from moderate liver damage 64–104 hr after a paracetamol overdose were investigated. In all patients there were substantial elevations in serum bilirubin and aspartate aminotransferase (Table 1) and the prothrombin time became 12 or more sec prolonged (prothrombin time ratio >2.1) at the peak of the liver damage. They were 12 h fasted at the time of the test but were given at least 250 g of carbohydrate in the 24 h period before this in order to minimise the risk of hypoglycaemia.[2] One patient with viral hepatitis (Type A) in the acute phase, was also investigated. Informed consent was obtained prior to the investigations. The results were compared to a control group of volunteers (laboratory personnel; 4 male, 2 female) who were age matched but who were somewhat heavier than the patients (109.4 ± 2.2% compared to 94.1 ± 3.2% of ideal body weight). In no patient or control was there any previous evidence of liver disease or family history of diabetes mellitus and none were taking any drug known to affect carbohydrate metabolism.

Lactate Infusion Test

The lactate infusion test was a modification of the procedure described by Doar and Cramp,[5] and forearm utilisation of metabolites was also determined by the method of Jackson, Advani, Perry, Rogers, Peters, Day, and Pilkington.[6] On the morning of the test cannulae were inserted into a vein on the back of one hand for sampling of arterialised venous blood. This hand was warmed in an electric blanket throughout the period of the test. A further cannula was inserted into an ipsilateral antecubital vein and this cannula was connected to a Sage constant infusion pump (Orion Research Corporation, U.S.A.) for administration of sodium L(+) lactate (Sigma Ltd. UK) pH 7.4. Forearm blood flow was determined in the contralateral arm (after exclusion of the hand with a wrist cuff) by venous occlusion plethysmography using a mercury in rubber strain gauge (Devices Ltd. United Kingdom)[7] and from this arm venous blood samples were collected from a cannula inserted into an antecubital vein draining deep structures.

After insertion of all cannulae the patient was rested in a recumbent position for 15–30 min and encouraged to fall asleep. Two basal blood samples were collected and then a loading dose of 389 μmole kg^{-1} of lactate was injected followed by a constant infusion of sodium lactate 27.9 μmole kg^{-1} min^{-1} which was

Table 1. Clinical Details of Patients Investigated. The Liver Function Tests Shown are Those Recorded on the Day of the Test

Sex	Age	Wt (kg)	Amount of Paracetamol Ingested (g)	Time of Test (hr after ingestion)	Prothrombin Time (s prolonged)	Plasma Aspartate Aminotransferase (iu/1) < 50	Bilirubin (μ mole/l) <17	Albumin (g/l) 40
Paracetamol Patients								
M	55	58	50	72	7	>2500	39	42
M	19	70	15	84	36	1300	113	38
M	44	58	38	64	12	>2500	38	34
M	32	59	50	70	20	>2500	58	41
M	25	57	25	90	7	2450	63	40
F	22	56	40	104	14	2050	99	32
		The paracetamol patients were 94.1 ± 3.2% and controls 109.4 ± 2.2% of ideal body weight, (72 kgs)						
Viral hepatitis								
F	29	51	—	—	1	170	325	38

maintained for 40 min. The loading dose was calculated to raise lactate concentration to a level which could be maintained by the infusion assuming a lactate distribution volume of about 20% of body weight. Arterialised venous and venous samples were collected at 10 min intervals for 80 min and additional arterialised venous samples at 5 min intervals from 40-80 min. The infused lactate was sterilised by autoclaving and the concentration of L(+) lactate was checked chemically prior to use.

Estimations

Whole blood was deproteinised by addition of an equal volume (3 ml) of ice cold perchloric acid (0.63 mole l^{-1}) and the supernatant was neutralised with potassium hydroxide (1.78 mole l^{-1}). Lactate and pyruvate,[8] glucose[9] and 2-oxoglutarate[10] were determined by standard enzymatic techniques. Liver function tests were determined in the routine clinical chemistry laboratory using the SMA 12/60 apparatus. Plasma insulin was measured by a modification of the radioimmunoassay of Soeldner and Slone.[11]

Calculations

For paracetamol patients steady state lactate concentrations were never reached during the infusion period, a continued rise in concentration being observed. Furthermore the lactate concentration during the decay period fitted a double rather than a single exponential curve. The results have therefore been analysed using the two compartment analysis of Gibaldi and Perrier.[12] The 40–80 minute lactate concentrations were fitted to a curve $y = Ae^{-\alpha x} + Be^{-\beta x}$, by minimising the sums of the squares of the residuals. From this

$$\text{Volume of central compartment } (V_C) = \frac{k_o}{A\alpha + B\beta}.$$

where k_o is the infusion rate

$$\text{Volume of tissue compartment } (V_T) = \frac{V_C k_{CT}}{k_{TC}}$$

where k_{CT} and k_{TC}, the rate constants from central to tissue compartment and from tissue to central compartment respectively are given by

$$k_{TC} = \frac{\alpha\beta}{k} \quad \text{and} \quad k_{CT} = \alpha + \beta - k_{TC} - k$$

where, k the rate of elimination and metabolism from the central compartment, is given by $k = 2\beta(A + B)/A\beta + B\alpha$

$$\text{Volume of distribution of lactate } V_D = V_C + V_T$$

For control subjects the lactate decay curve was single exponential and thus for controls the 40–80 min lactate concentrations have been fitted to the curve $y = Ae^{-\alpha x}$

$$\text{In this case } V_D = \frac{k_o}{Ak}$$

where k_o is the infusion rate and k is the elimination constant α. The endogenous production rate of lactate was calculated from $k \times V_D \times L_F$ where L_F is the fasting lactate concentration.

Statistical Methods

Mean values ± SEM are quoted. P values refer to the Student's t test or paired t test where appropriate.

RESULTS

Fasting Lactate Concentrations

There was a twofold increase in fasting lactate concentrations in paracetamol patients when compared to control subjects ($P < 0.02$; Fig. 1), and in the viral hepatitis patient a three fold increase was found (Table 2).

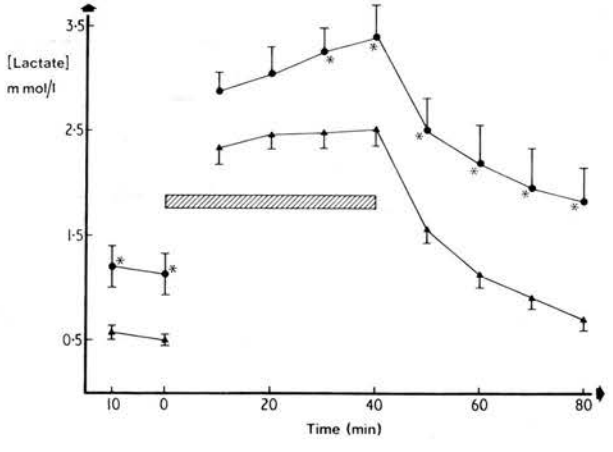

Fig. 1. Sequential arterialised-venous lactate concentrations in patients with paracetamol induced liver damage (●) and control subjects (▲) (•P < 0.05). The hatched area represents the lactate infusion.

Table 2. Some Indices of Lactate Metabolism in Patients With Liver Damage and Controls

	Paracetamol Patients (6)	Viral Hepatitis Patient (1)	Controls (6)
Fasting lactate concentration (L_F) m mole l^{-1}	1.17 ± 0.19*	1.77	0.53 ± 0.05
40 min lactate increment mmole l^{-1}	2.22 ± 0.15	2.23	1.95 ± 0.09
Lactate elimination constant (k)	0.029 ± 0.002†	0.036	0.063 ± 0.002
Lactate distribution volume (V_D) mls kg^{-1}	255 ± 13*	—	222 ± 10
Endogenous production rate μmole kg^{-1} min^{-1}	8.6 ± 1.1	—	7.4 ± 0.7
A	0.71 ± 0.13	—	2.0 ± 0.04
B	1.51 ± 0.13	—	—
α	0.158 ± 0.033	—	(see k above)
β	0.021 ± 0.003	—	—
A, B, α, and β are parameters of the curve $y = Ae^{-\alpha x} + Be^{-\beta x}$			

*$P < 0.05$.
†$P < 0.001$.

Blood Lactate Decay Curves

In control subjects, the blood lactate concentrations after cessation of the infusion were best fitted by single exponential curves but in paracetamol patients the decay curve was double exponential in character (Fig. 2). The lactate elimination constant (k) was significantly prolonged in paracetamol patients when compared to controls (0.029 ± 0.002 compared to 0.063 ± 0.002; $P < 0.001$; Table 2).

We have previously shown a relationship in control subjects between absolute lactate concentrations at the start of the lactate decay curve (L_θ) and the fractional rate removal constant (k) ($\log_e k = -2.43 - 0.132 L_\theta$; Woll and Record[13]). It is thus of paramount importance when comparing fractional rate removal constants for decay to commence from similar absolute lactate concentrations. In the paracetamol group however, the mean L_θ was 0.91 mole l^{-1} higher than in the control group (Fig. 1) and this corresponds to a reduction in k of 0.007 $min.^{-1}$ This is insufficient to account for the observed difference between the control group and k of the paracetamol group.

Lactate Distribution Volume

In the control subjects a steady state lactate concentration was obtained during the course of the infusion so that the 20, 30, and 40 min values were very similar (Fig. 2). In paracetamol patients however, due to an

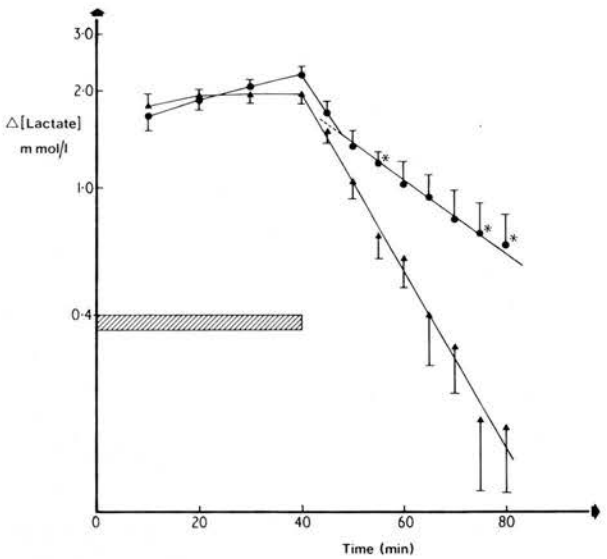

Fig. 2. Sequential incremental arterialised-venous lactate concentrations Symbols as in Fig. 1.

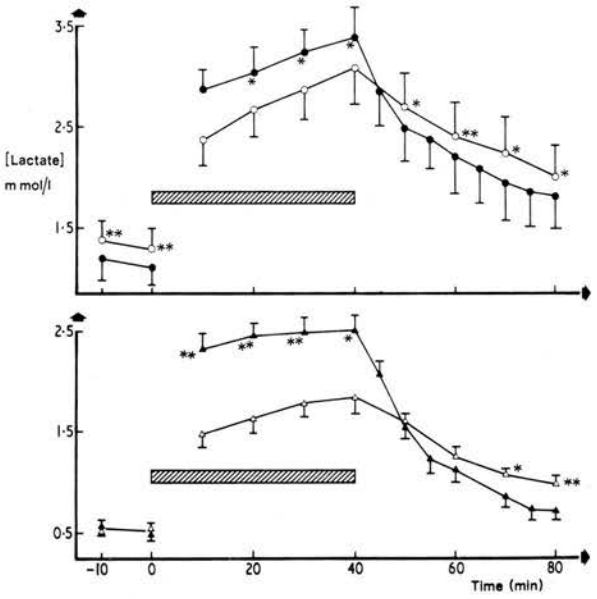

Fig. 3. Arterialised-venous (closed symbols) and venous (open symbols) lactate concentrations in paracetamol patients and control subjects. Symbols as in Fig. 1. (*P < 0.05; **P < 0.01).

Table 3. Forearm Consumption of Lactate

	Mean Forearm Muscle Flow*	Consumption†	Skeletal Muscle Lactate Uptake‡ in a Man of Mean Weight for Group	Percentage of Lactate Metabolised by Skeletal Muscle§
	mls 100 ml muscle^{-1} min^{-1}	μmol 100 ml forearm muscle^{-1} min^{-1}	mmole min^{-1}	
Paracetamol Patients (0–40 min)	2.9 ± 0.4	1.58 ± 0.16	0.67	39%
Controls (0–40 min)	2.3 ± 0.1	1.56 ± 0.11	0.79	17%

*Muscle flow = 0.47 × total flow + 0.83.

†Incremental AV difference × muscle blood flow.

‡Assumes forearm 60% muscle, 30 kg muscle 70 kg^{-1} man and muscle density 1.016.

§Skeletal muscle lactate uptake/rate constant for removal × mean weight for group.

increase in T½, steady state concentration was never attained, the mean incremental blood lactate concentration at 40 min being 18% higher than at 20 min. In calculating the distribution volume of lactate no assumptions have therefore been made concerning steady state conditions during the infusion. V_D was marginally significantly higher in the paracetamol patients (255 ± 13 mls kg^{-1} compared to 222 ± 10; $P < 0.05$; Table 2). The notional total volumes however (15.3 and 16.0 l respectively) were not significantly different.

Forearm Arterio-Venous Differences

In both paracetamol patients and control subjects there was substantial and significant (paired t test; Fig. 3) consumption of lactate in the forearm during the infusion period. However, after cessation of the infusion arteriovenous differences reversed and the forearm became a producer of lactate. For paracetamol patients AV differences were significantly negative at 50–80 min ($P < 0.01$) and for control subjects at 70 and 80 min ($P < 0.01$). In paracetamol patients fasting forearm venous blood lactate concentration was significantly greater than arterial ($P < 0.001$) whereas in control subjects fasting arterial and venous samples were similar (Fig. 3).

Forearm Consumption of Lactate

The consumption of lactate in the forearm during the course of the lactate infusion period was calculated from the mean forearm blood flow and the mean incremental arteriovenous difference of lactate. Since the mean total forearm blood flow was greater in the paracetamol patients than the controls and since the proportion of total forearm flow supplying muscle diminishes as the flow increases, a correction was applied to the values as described by Cooper, Edholm and Mottram.[14] Total lactate consumption in muscle was estimated as previously described.[13] Lactate consumption in the forearm was substantially greater in the paracetamol patients than controls (Table 3).

Blood Pyruvate Concentrations

Blood pyruvate concentrations reflected blood lactate values. During the infusion periods there was significant consumption of pyruvate in the forearm but during the decay periods arterial and venous concentrations were similar, (Fig. 4).

Lactate/Pyruvate ratios were significantly more reduced in the infusion period than during fasting but paracetamol patients and control subjects behaved similarly. After cessation of the infusion the ratios returned to the preinfusion values.

Glucose Insulin, and 2-Oxoglutarate Concentrations

Mean fasting blood glucose concentrations were slightly but not significantly lower in paracetamol patients than controls (4.36 ± 0.41 compared to 4.92 ± 0.16 mole l^{-1} respectively), but in one parace-

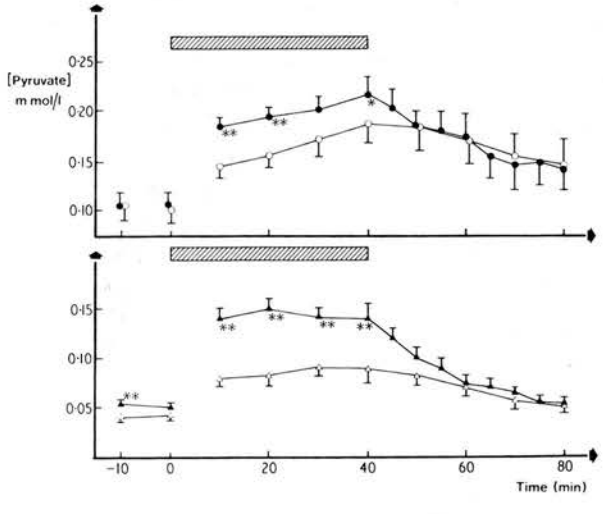

Fig. 4. Arterialised-venous and venous pyruvate concentrations. Symbols as in Fig. 3.

tamol patient a value of only 2.90 mole l^{-1} was found. There was no significant change in blood glucose concentration during the infusion or decay periods in either group.

Fasting insulin concentrations were identical in the paracetamol (6.8 ± 0.7) and control (6.8 ± 0.7 μU ml^{-1}) groups and in two control subjects there was no change in insulin concentrations through the infusion and recovery periods.

Fasting 2-oxoglutarate concentrations were significantly increased in paracetamol patients (0.016 ± 0.001 compared to 0.011 ± 0.001 respectively; $P < 0.01$) and this increase was maintained throughout the period of the test.

DISCUSSION

The limitations and assumptions of the lactate infusion test described by Doar and Cramp[5] as a measure of lactate metabolism have been commented on in detail by these authors and in a previous paper.[13] In particular it is assumed that lactate load does not affect the endogenous production rate of lactate and that lactate is distributed in a single compartment. However, for patients with paracetamol induced liver damage the lactate decay curve was double exponential in character and this may indicate a two compartment system. Furthermore, steady state conditions were never achieved. The results have therefore been analysed using a two compartment model which does not assume steady state conditions during the infusion and from this the volume of distribution and lactate elimination rate were obtained.

In both control subjects and patients with paracetamol-induced liver damage the lactate distribution volume was similar to the anticipated extracellular fluid volume. This is somewhat less than that found by Searle and Cavalieri[15] (49.9% body weight) using isotopic techniques but is similar to that reported by Doar and Cramp.[5] Yudkin and Cohen[16] found rather higher values in the rat, a mean value of 337 ml kg^{-1} being quoted.

The lactate elimination rate was markedly reduced in patients with paracetamol-induced liver damage suggesting that the liver is of major importance in removal of lactate from the circulation. This diminished elimination resulted in a significant increase in fasting lactate concentration in the paracetamol group.

The disposition of infused lactate in a two compartment system profoundly affects the calculated parameters of lactate metabolism and it is thus essential to employ the correct model in particular circumstances. Adoption of the single compartment model of Doar and Cramp[5] for paracetamol patients resulted in a calculated volume of lactate distribution which was spuriously high and which clearly did not fit with the experimental findings. It was for this reason that this model was rejected in favour of the two compartment model of Gibaldi and Perrier.[12]

There was no change in blood glucose concentration during the infusion period and it therefore seems likely that any glucose formed from lactate by the liver would either have been incorporated into glycogen or have replaced the normal post-absorptive output of glucose by the liver.[17]

The changes in lactate metabolism in paracetamol patients are likely to be the result of the liver damage rather than any specific effect of paracetamol since paracetamol and its metabolites are completely excreted within 48 hr of ingestion unless fulminant hepatic failure supervenes.[18] In contrast to the paracetamol and hepatitis patients, patients with cryptogenic cirrhosis have a normal fasting lactate concentration. Lactate elimination however is also significantly decreased.[13]

Site of Lactate Removal

Over the 40 min infusion period there was substantial overall consumption of lactate in the forearm. Assuming the forearm consists of 60% muscle and that there was 30kg of muscle in a 70 kg man, it can be calculated for the paracetamol patients that during the infusion period approximately 39% of infused lactate was metabolised in voluntary muscle (Table 3). For control subjects approximately 17% was metabolised in voluntary muscle. This difference suggests a compensatory increase in muscle uptake in the presence of liver damage. The ability of voluntary muscle to take up lactate has previously been recognised particularly during exercise. Freyschuss and Strandell[19] for instance estimated that between 5% and 12% of lactate produced by the arms or legs exercising separately was taken up in the resting limbs. More recently Hermansen and Stensvold[20] found a higher removal rate of lactate after severe exercise if this was followed by submaximal exercise rather than complete rest. From these experiments they concluded that skeletal muscle was the main site for lactate removal during exercise. More recently Ahlborg, Hagenfeldt and Wahren[21] have shown that 35% of an infused lactate load is taken up in resting voluntary muscle. Two possible routes are available for disposal of lactate taken up in voluntary muscle. Complete oxidation probably accounts for the majority but a small proportion may be released as alanine. In this context Pozefsky and Tancredi[22] found that less

than 7% of pyruvate infused into the brachial artery was released from the forearm as alanine.

Relevance to Clinical Lactic Acidosis

Previous studies have suggested that liver disease is an important pathogenetic factor in lactic acidosis (see Cohen and Woods[23]). The present studies show that when there is substantial liver damage the ability of the body to dispose of a lactate load is severely deranged. However, in the absence of hypotension, severe hyperlactataemia is an unusual accompaniment of the most severe form of liver damage—fulminant hepatic failure[3] and this is probably due to the low lactate load normally presented to the liver. In contrast when the body is presented with a massive lactate load, as occurs when hypotension supervenes, marked hyperlactataemia and systemic acidosis result.[24]

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Short Communication

THE PROGNOSTIC VALUE OF THE CUTANEOUS DELAYED HYPERSENSITIVITY RESPONSE TO 2-4 DINITROCHLOROBENZENE IN GASTROINTESTINAL CANCERS

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Received 21 January 1974. Accepted 6 February 1974

IT IS frequently difficult to predict the likely course of patients with malignant diseases. With increasing awareness of the role of "host" resistance in this context, many workers have been stimulated into searching for immunological methods for assessing the strength of this resistance and its role in prognosis.

As recent evidence suggests that the major part of the immunological response to neoplasia is cellular (Hellström and Hellström, 1969; Burnet, 1970), it is obviously important to evaluate cell mediated reactions in patients with cancer. The cutaneous delayed hypersensitivity response to naturally occurring antigens has been used as an *in vivo* assessment of cellular immunity in subjects with malignant diseases (Solowey and Rapaport, 1965; Hughes and Mackay, 1965). These workers have demonstrated decreased responsiveness to the antigens employed in patients with advanced neoplasia. Since "positive" results rely upon prior exposure to the antigen, a "negative" result to one or more antigens may be of little relevance in determining anergy. This defect can be overcome by sensitizing patients to artificial allergenic chemicals to which they have not previously been exposed. One such chemical allergen is 2-4 dinitrochlorobenzene (DNCB) which will induce 95% sensitization in normal individuals (Eilber and Morton, 1970). In recent studies using this chemical, these authors have shown that patients who are anergenic to DNCB have a high incidence of early recurrence of malignant disease following surgical resections.

The present study was undertaken to investigate the relationship between the initial delayed hypersensitivity reaction to DNCB, following sensitization, in patients with gastrointestinal tumours and their subsequent survival.

MATERIALS AND METHODS

Seventy patients with gastrointestinal tumours were studied during this investigation. Table I itemizes the tumours studied. There were 38 males and 32 females and their ages ranged from 36 to 81 years (mean 63 years). No patients receiving x irradiation, chemotherapeutic agents or steroids were included. Patients who were found to be uraemic, who died during the operation or in the immediate post-operative period were also excluded.

Twenty "control" patients were obtained from the same hospital population. All were admitted for large bowel investigation and their consent was obtained before being included in this study. Their investigations

TABLE I.—*Sites of Carcinoma in Patients Studied*

Sites of carcinoma	No.
Rectum	24
Caecum and colon	18
Stomach	17
Oesophagus	5
Pancreas	5
Gallbladder	1

revealed no obvious pathology in 7 and diverticulosis in the remaining 13. There were 9 male and 11 female patients in this " control " group, their ages ranging from 44 to 80 years (mean 64 years).

DNCB sensitization and testing.—The technique employed was adapted from that of Ratner, Waldorf and Scott (1968). Patients were sensitized by the application of 4000 μg of DNCB dissolved in acetone and applied to a 3 cm^2 area of skin on the volar aspect of the forearm. An occlusive polyethylene dressing was put over the site and left in position for 4 days.

Sensitivity tests were carried out by applying 4 Al patch test squares (Imeco A.B. Stockholm) each impregnated with a different strength of DNCB in acetone (200, 100, 40 and 2 μg). These squares were applied on the opposite forearm *at least* 14 days after sensitization. The test was read at 48 h, a " positive " response being recorded when there was erythema and induration under the whole of the patch test area.

Patients were then graded according to their sensitivity as follows:

Negative	no reaction to any strength
i	sensitive to 200 μg *only*
ii	sensitive to 200 and 100 μg
iii	sensitive to 200, 100 and 40 μg
iv	sensitive to ALL strengths of DNCB tested.

Follow-up.—All patients were seen at regular intervals on an out-patient basis following their treatment in hospital. The exact date of death was recorded where appropriate.

RESULTS

The results of the delayed hypersensitivity reactions to DNCB in the cancer patients and controls are recorded in Table II. Grades i and ii have been grouped together because of the small numbers in these groups.

TABLE II.—*Delayed Hypersensitivity Responses to DNCB in Cancer and Control Patients*

Patients	Negative	I and II	III	IV	Total
Cancer	21	15	20	14	70
Control	1	5	10	4	20

The cancer patients were divided almost equally between the 4 groups with no significant difference between the proportions in each group ($\chi^2 = 6{\cdot}21$, d.f. $= 3$, P 0·1). All but one of the control patients showed a delayed hypersensitivity reaction to DNCB and the majority were graded iii.

If the cancer and control patients were graded as either " positive " or " negative " (Table III) there was a highly significant difference between them $P \approx 0{\cdot}02$ (Fisher's exact test).

TABLE III.—*Response to DNCB in Cancer and Control Groups Expressed as Positive or Negative*

	DNCB response	
Patients	Negative	Positive
Cancer	21	49
Control	1	19

When the cancer patients' survival was compared with their initial DNCB sensitivity grading (Fig.) there was a striking correlation between their relative anergy and their subsequent progress. All but 2 patients in the Negative and Grade i categories were already dead at the time of the review, the longest recorded survival being only 9 months, whereas in Grades ii, iii and iv there had been only 2 deaths and patients had survived for up to 12 months (which was the maximum period possible at the time of this review).

In Table IV the initial DNCB responses have been compared with the 6-month mortality figures, excluding all patients who were alive but who had not yet reached 6 months of follow up. There was a significant difference between these

TABLE IV.—*6-Month Mortality Figures with Corresponding Initial Response to DNCB*

	Initial response to DNCB				
At 6 months Patients	Negative	I & II	III	IV	Total
Dead	17	9	2	0	28
Alive	4	4	14	12	34
	81%	69%	12%	0%	45%

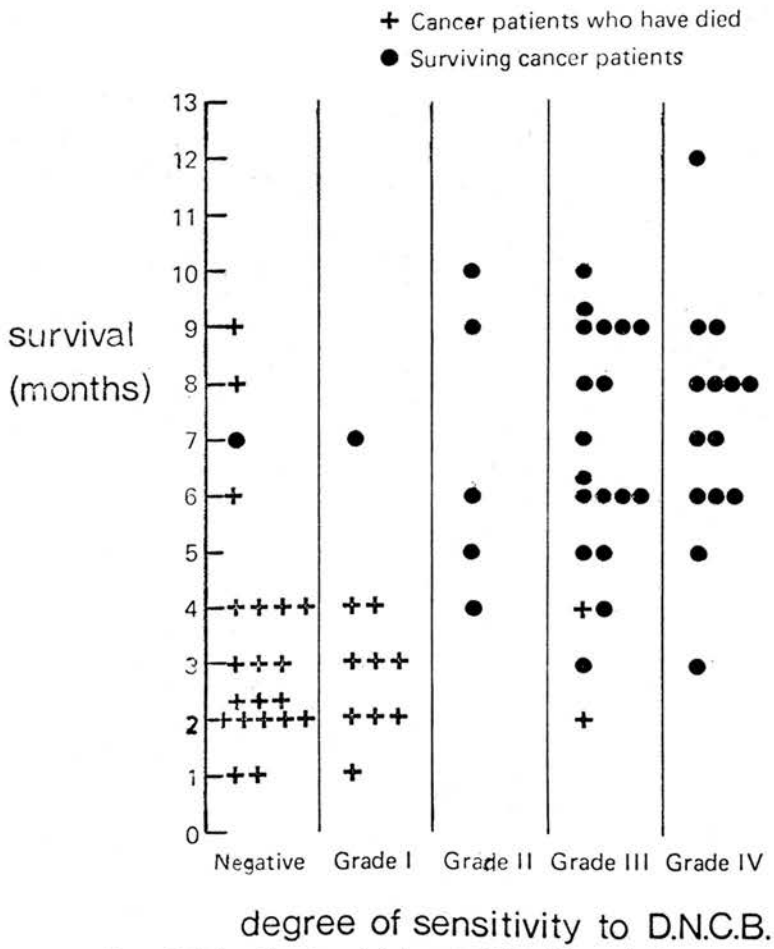

FIG.—Relationship of sensitivity to DNCB with survival.

groups ($\chi^2 = 30{\cdot}68$, $P < 0{\cdot}001$) with a linear trend ($\chi^2 = 28{\cdot}36$, d.f. $= 1$, $P < 0{\cdot}001$) from which there was no significant deviation ($\chi^2 = 2{\cdot}32$, d.f. $= 2$, $P > 0{\cdot}2$).

Overall 45% of the patients had died within the first 6 months after their initial hospital admission and assessment. The prognosis for Grade iv responders was the most favourable, with a progressive decline with diminished initial response.

DISCUSSION

The present study has demonstrated that there is a linear relationship between the strength of the delayed hypersensitivity response to an exogenous allergen (DNCB) and the short-term prognosis in patients with gastrointestinal cancers. A striking finding was that patients who were anergic had only a 20% chance of surviving for 6 months, leading us to question whether radical surgery is ever justified in such patients, unless something can be done to reverse their " anergic " state.

Whether this test will be of value in predicting long-term survival will have to await further follow-up studies. Nevertheless, the accurate prognostic assessment of patients with cancer could be valuable in decisions regarding treatment regimes.

We would suggest that the routine use of this test in patients with gastrointestinal cancer could be a useful addition to current clinical assessment and might lead to changes in management.

We should like to thank Professor Johnston for his help and encouragement during this work, the Photographic Department for production of the illustrations, Mr Poole for help in the formulation of the DNCB, Mrs M. Foster for the secretarial help, The Cancer Research Campaign for supporting Mr G. Bone during this work and to all the patients who willingly participated in this study.

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Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer

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J Clin Pathol 1985;**38**:1225–1228

Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer

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SUMMARY Epidermal growth factor receptors are present in some breast cancers in man, and there is an inverse relation to oestrogen receptor state. We assessed the presence of epidermal growth factor receptors as a single prognostic indicator in a series of breast tumours by comparing this with the Bloom and Richardson scores for these tumours. One hundred and eight ductal tumours were examined for epidermal growth factor receptors by radioligand binding. There was a significant ($p < 0.01$) correlation between the presence of the growth factor receptor and poor prognosis as assessed by the Bloom and Richardson score, suggesting that epidermal growth factor receptor state could be a useful prognostic marker. Epidermal growth factor receptor state was not significantly correlated with the lymph node state but showed a tendency to be associated with large tumours.

Epidermal growth factor receptors have been found in 35% of specimens of human breast cancer, and there is a negative association with oestrogen receptor state.[1] Epidermal growth factor receptors have been shown in other tumours including those of the lung,[2] bladder,[3] brain,[4] ovary, and uterus.[5]

In culture epidermal growth factor is a mitogen for breast tissue[6] that is both benign and malignant.[7,8] It is an essential part of chemically defined serum free media for breast cancer cells.[9] Functional epidermal growth factor receptors occur in the developing breast[10] and have been shown in breast cancers both as cell lines and as biopsy specimens.[11,12]

Epidermal growth factor receptors closely resemble the oncogene v-erb-B[13] and share with the oncogenic retroviruses the ability to cause phosphorylation of tyrosine residues of intracellular proteins (an unusual site of such activity). The confirmation of this similarity has led to the hypothesis that the signal induced by epidermal growth factor receptors might be responsible for uncontrolled growth of the cell. The erb-B product lacks the external binding site for epidermal growth factor, which may thus leave the receptor in a permanently activated state. This hypothesis of an overdrive mechanism remains to be proved.

The growth factors binding to the epidermal growth factor receptors may be produced locally by autocrine or paracrine mechanisms, and it has been suggested that the ability of a cell to both produce growth factors and express their receptors on the cell surface is a malignant phenotype.

There have been many attempts to correlate the histological appearance of tumour material, clinical data, and operative findings to ultimate outcome. The Bloom and Richardson scoring system[14] based on three histological criteria is a well recognised discriminator. It can be refined into a more accurate system by incorporating details such as lymph node state (which is the best single prognostic factor at present) and size of tumour.[15] Although oestrogen receptors are useful in predicting likely response to treatment and survival after first relapse,[16] they are not good at predicting time to relapse. Tumours positive for oestrogen receptors tend to relapse in bone and soft tissues and those negative for oestrogen receptors recur in the liver and brain.[17] The oestrogen receptor state did not greatly increase the predictive power of either the Nottingham index[15] or the recently reported analysis from Edinburgh.[18]

We investigated the relation between epidermal growth factor receptor state and prognostic factors such as the Bloom and Richardson grade, size of tumour, and lymph node state. When data of recurr-

Accepted for publication 6 August 1985

ence become available more direct evidence of a correlation with outcome will be sought.

Material and methods

One hundred and eight specimens of breast cancer were obtained fresh from surgery and processed immediately.[1] All the tumours had epidermal growth factor receptors assessed by a radioligand assay, and on 48 of these immunohistochemical analyses were subsequently performed. Briefly, a membrane preparation was obtained by differential centrifugation and aliquots incubated with iodinated epidermal growth factor (0·6 nmol/l, specific activity 2·96 MBq (80 μCi)/μg) with or without known amounts (0·01–1000 ng/ml) of unlabelled epidermal growth factor to displace the binding. From these data a Scatchard plot was derived. Positive tumours gave binding data with a mean dissociation constant of 1·7 nmol/l (range 0·7–2·3) with between 4 and 47 fm/mg protein bound. Sixty two tumours were assessed by a two point assay in which duplicates of membrane preparations were incubated with iodinated epidermal growth factor either with or without 800 ng unlabelled epidermal growth factor/ml. A displacement of greater than 75% was taken as positive.

Oestrogen receptors were measured on the tumour samples by a dextran coated charcoal method in accordance with the recommendations of the United Kingdom Quality Assurance scheme. Immunohistochemical analyses were also performed on 48 sections snap frozen at the time of collection. A monoclonal antibody against the external binding site of the epidermal growth factor receptors (gift of Dr MD Waterfield) was used and stained by the indirect peroxidase method. The working dilution of the primary antibody was 1/50 and that of the secondary (Dako 261) 1/20. Sections of placenta (rich in epidermal growth factor receptors) were used as positive controls and preincubation of the antibody with purified receptor (prepared from A-431 cells, gift from Dr P Parker) before staining as negative adsorption controls. An irrelevant antibody of the same class (2a) was also used as a negative control. Sections were graded on a +++, ++, +, 0 basis by two observers and those given a +++ or ++ grade by either observer counted as positive. Routine histological examination was performed after fixation of the samples with formalin and impregnation with wax. Sections were stained with haematoxylin and eosin and assessed for degree of tubular formation (differentiation), hyperchromatic and mitotic figures, and nuclear pleomorphism. These were scored on a 1–3 basis and the scores totalled to give a 3–9 score. This scoring was performed with no knowledge of epidermal growth factor or oestrogen receptor state. Grade I comprised scores 3–5; grade II, 6–7; and grade III scores 8–9. Patients with tumours exhibiting a high score were likely to relapse and die earlier.[14] This scoring system was used only for ductal carcinomas; lobular, mucinous, and other rare tumours (a further 11) were not graded or included in this analysis as the Bloom and Richardson scoring system does not allow for their assessment.

Estimates of elastosis and round cell infiltration were also scored on a 1–3 basis, and measurements of size of tumour and lymph node state (when available) were also recorded. The data were analysed by using a logistic transformation of proportions, both for single variable analysis and for a multivariate analysis to examine the effect of all the variables in combination.[19]

Table 1 *Relation between epidermal growth factor receptor and oestrogen receptor*

Epidermal growth factor receptors	*Oestrogen receptor*		*Total*
	Positive	*Negative*	
Positive	3	42	45
Negative	46	17	63
Total	49	59	108

χ^2 = 43·9 with 1 df; p = <0·001.

Table 2 *Relation between immunohistochemical and radioligand epidermal growth factor receptor assays*

Radioligand assay	*Epidermal growth factor receptor on immunohistochemical assay*		
	Positive	*Negative*	*Total*
Epidermal growth factor receptors:			
Positive	21	5	26
Negative	2	20	22
Total	23	25	48

Specificity of immunohistochemistry = 91%.
Sensitivity of immunohistochemistry = 81%.

Results

One hundred and eight tumours were included in

Table 3 *Epidermal growth factor receptor and Bloom and Richardson score*

	Bloom and Richardson grade			
	I	*II*	*III*	*Total*
Epidermal growth factor receptors:				
Positive	2	11	32	45
Negative	10	25	28	63
Total	12	36	60	108
Positive for epidermal growth factor receptors (%)	17	31	53	

χ^2 for logit linear trend in proportions = 8·64 with 1 df; p = 0·003.

Table 4 *Relation between size of tumour and epidermal growth factor receptor state*

	Size (cm)				
	<2	*2·1–3·5*	*3·6–5*	*>5*	*Total*
Epidermal growth factor receptors:					
Positive	8	22	7	8	45
Negative	21	28	9	5	63
Total	29	50	16	13	108
Positive for epidermal growth factor receptors (%)	28	44	44	62	

χ^2 for logit-linear trend in proportions = 3·97 with 1 df; p = 0·046.

this analysis. Forty five were positive for epidermal growth factor receptors by radioligand assay. The previously reported inverse relation between epidermal growth factor receptors and oestrogen receptors was again noted. Forty two of the 45 tumours positive for epidermal growth factor receptors were negative for oestrogen receptor state and 46 of 49 tumours positive for oestrogen receptor state were negative for epidermal growth factor receptors; three were positive for both and 17 doubly negative (Table 1).

There was good agreement between radioligand and immunohistochemical techniques. Of the 48 tumours assessed both ways, 41 showed agreement (Table 2). The specificity (defined as one minus the probability of a false positive) was 91% and the sensitivity (one minus the probability of a false negative) was 81%. There was a significant association between tumours positive for epidermal growth factor receptors and high Bloom and Richardson scores. When assessed in the absence of other factors (Table 3) this association was significant ($p < 0·01$).

Table 4 shows that cancers positive for epidermal growth factor receptors tended to arise from a population of larger tumours; and Table 5 shows that spread to the lymph nodes was not conclusively related to epidermal growth factor receptor state, although this may have been due to the low number of tumours categorised for lymph node state. Elastosis and round cell infiltration were not significantly related to epidermal growth factor receptor state.

To examine the relation of epidermal growth factor receptors with the various predictors for a poor prognosis a logistic discriminant analysis was performed. In this the actual tumour size (rather than the categorisation of Table 4) was used. Table 6 shows the contribution of each variable to the discriminant function. Only epidermal growth factor receptors were significantly related to the Bloom and Richardson score.

Table 5 *Relation between epidermal growth factor receptor state and lymph node state*

	Lymph node state		
	Affected	*Unaffected*	*Not measured*
Epidermal growth factor receptors:			
Positive	21	9	15
Negative	14	15	34

χ^2 = 2·05 with 1 df.

Table 6 *Relation of epidermal growth factor receptor to size of tumour and Bloom and Richardson score*

Source	*Deviance*	*df*	*p*
Log diameter:			
without B and R grade	3·51	1	ns
after fitting B and R	1·56	1	ns
Bloom and Richardson:			
without fitting log diameter	8·64	1	0·001
after fitting log diameter	6·70	1	0·001
Residual	136·5	105	
Total	146·71	107	

p is the probability of positive epidermal growth factor receptor $\log \frac{P}{1-P} = -2·958 + 0·518$ log diameter (cm) (SE 0·419) + 0·833 Bloom and Richardson score (SE 0·338).

The follow up time was too short to analyse clinical relapse rates, but three of the four patients relapsing in under 15 months were positive for epidermal growth factor receptors.

Discussion

The Bloom and Richardson score is a good indicator of likely risk of recurrence[14] and when combined with the presence of lymph node metastases and size of tumour (as in the Nottingham index[15]) is a good predictor of likely outcome. This index was initially derived retrospectively but has recently been shown to work in a prospective manner.[20] The addition of staining intensity using a monoclonal antiepithelial antibody (NCRC 11) has been reported to increase further the accuracy of the index.[21]

Positivity for epidermal growth factor receptor seems to be an indicator of poor prognosis in breast cancer. This was suggested by the previous finding of an inverse relation between oestrogen receptor and epidermal growth factor receptors[1]: tumours positive for epidermal growth factor receptors arose from tumours negative for oestrogen receptor state (therefore indicating bad prognosis). Why epidermal growth factor receptors should be present in this group of tumours is unclear. Other workers confirmed the presence of these receptors in specimens of breast cancer.[22,23]

Possibly, the expression of growth factors is akin to the secretion of α fetoprotein or carcinoembryonic antigen and signifies that dedifferentiation is

occurring. Possibly, this group of tumours uses the epidermal growth factor receptors as the growth initiation signal either by overproduction of normal receptors or even by activation of the cellular oncogene. The second alternative is unlikely as c-erb has not been isolated from breast tumours and, also, as the predicted structure of the erb B product lacks the binding site for external growth factor.

This study showed a correlation between presence of epidermal growth factor receptors and high Bloom and Richardson grades that is independent of size of tumour, lymph node state, elastosis, and round cell infiltrate. One drawback of this study is that the epidermal growth factor receptors were compared with a predictive index and not an absolute such as death or recurrence. These data will emerge as the cohort of patients are followed up in the clinic. It will be interesting to see which combination of predictor variables will prove the best estimate of prognosis.

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Published by British Medical Association, Tavistock Square, London WC1H 9JR, and printed in England by Eyre & Spottiswoode Ltd, Thanet Press, Margate

David Appleton

Reprinted from AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY
Vol. 39, No. 2, September 1973 © The Wistar Institute Press 1973

Interactive Use of Computer Models in Teaching Population Genetics

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KEY WORDS Gene frequency · Population genetics · Genetic drift · Equilibrium · ABO · Computer models · Teaching.

ABSTRACT A technique using a computer with a graphical display unit to teach students the effects of genetic drift, selection and migration is described. Both diallelic and triallelic loci are discussed. The Fokker-Planck equation is used as the mathematical model of the genetic system, and its validity as an approximation in this context is demonstrated by an investigation into selection at the ABO locus.

An appendix contains a derivation from the Fokker-Planck equation of the formula used in the paper for the gene frequency distribution at a multiallelic locus in equilibrium under selection and migration.

The dispersive effect of random genetic drift and its prevention by directional forces such as selection and migration may prove difficult phenomena to communicate to students of anthropological genetics. It is often impossible within a given syllabus to use experimental material suitable to illustrate population gene frequency behaviour; the mathematics involved in the theoretical treatments in textbooks and research articles is sufficiently abstruse to deter almost all students, and the results contained in such sources are necessarily incomplete. A compromise solution was found by Crosby ('61) who used Monte-Carlo simulation techniques, but even with a computer, results in a comprehensible form are not available sufficiently quickly since it takes many successive simulations to build up a probability distribution. Arguably, the time spent in this pursuit is not wasted, as it can give the students an appreciation of what a probability distribution actually represents, but for complicated systems with many parameters the ability to investigate the effects and interactions of these parameters is lost.

An alternative approach is to teach students about probability distributions by using a pin-board or by picking coloured balls from a bag and to model the genetic system by the Fokker-Planck diffusion equation, described at length by Kimura ('64). In this way it is possible to investigate the behaviour of a diallelic locus under drift, to demonstrate the equilibrium produced by some values of selective coefficients of the genotypes, and to show the effects of altering population size or permitting immigration. Students should also be able to understand the influences exerted by the various factors at a locus where three alleles are present. If the probability distribution produced by solving the relevant diffusion equation is drawn directly on a graphical display unit by the computer program, intelligible results are available very quickly after the program has been given the necessary system parameters.

The models

I. Kimura ('70) has given the distribution $\phi(x, t; p)$ of the gene frequency x at a diallelic locus under random genetic drift, after t generations, as

$$\phi(x, t; p) = \sum_{i=1}^{\infty} p(1-p)i(i+1)(2i+1)\cdot F(1-i, i+2, 2, p)\cdot F(1-i, i+2, 2, x)\cdot \exp(-i(i-1)t/4N), 0 < x < 1,$$

where N is the effective population size,

p the initial gene frequency, and F(.,.,.,.) the hypergeometric function. This form of the solution is suitable for computer evaluation, and the graph for any N, p and t can be rapidly produced. The probability of fixation is found by numerical quadrature.

II. Li ('55) gives the probability density function for the gene frequency x at a diallelic locus in an unfixed population of effective size N as

$$\phi(x) = \frac{C\bar{w}^{2N}}{x(1-x)}$$

where C is a constant such that $\int_0^1 \phi(x)dx = 1$. If the fitnesses of the genotypes AA, Aa and aa are s_1, s_2 and s_3 and the frequencies of A and a are x and y $(= 1 - x)$ then the mean fitness $\bar{w} = s_1x^2 + 2s_2xy + s_3y^2$. This distribution may also be calculated rapidly. It is important to remember that it is only valid for $\frac{1}{2N} \leq x \leq 1 - \frac{1}{2N}$. The corresponding expression if migration also occurs at a rate m per generation from a population where A has frequency $\bar{x}$ is

$$\phi(x) = \frac{C'\bar{w}^{2N}}{x^{1-4Nm\bar{x}}(1-x)^{1-4Nm(1-\bar{x})}}$$

For given values of N, m, $\bar{x}$, s_1, s_2, and s_3 this distribution may be plotted almost immediately.

III. Wright ('49) has given the general result for a k-allelic locus under selection and migration. A derivation of this rather neglected formula, from the diffusion equation, is given in the APPENDIX. It is

$$\phi(\underline{x}) = \frac{C\bar{w}^{2N}}{\prod_{i=i}^{k} x_i^{1-4Nm\bar{x}_i}}$$

extending the notation used above in a natural manner.

For the triallelic case $(k = 3)$, if s_{ij} is the fitness for genotype A_iA_j and $s_{ij} = s_{ji}$, the 10 parameters of the system are N, m, $\bar{x}_1$, $\bar{x}_2$, s_{11}, s_{12}, s_{13}, s_{22}, s_{23}, and s_{33}. In this case the probability distribution for the gene frequencies is a bivariate distribution, and it was decided to represent it by a contour diagram in the triangle $0 < x_1 < 1$, $0 < x_2 < 1$, $0 < 1 - x_1 - x_2 < 1$, orienting this triangle after Brues ('63) in her investigation of the ABO locus.

The computer system

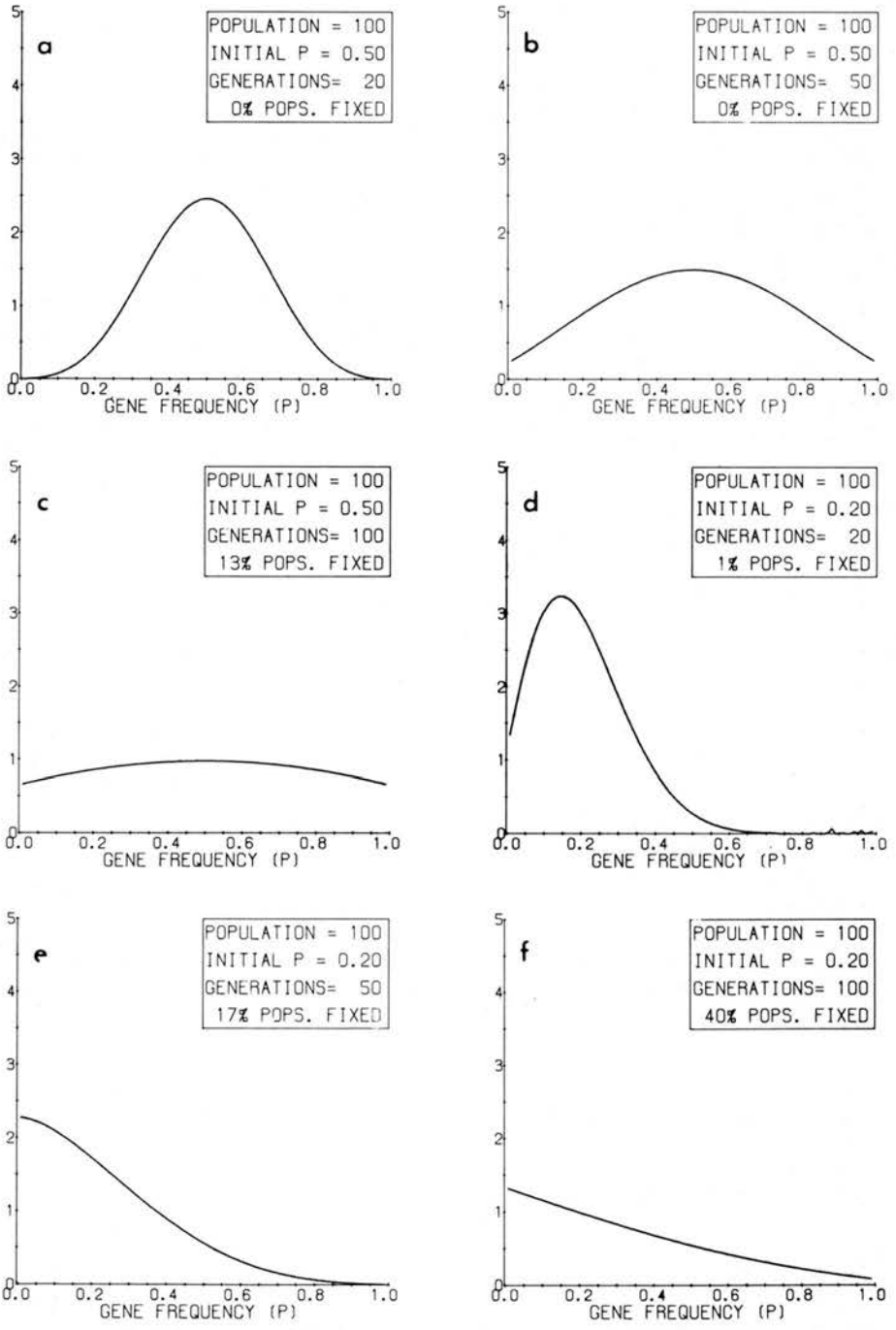
The models were run on an IBM 360/67 supporting the Michigan Terminal System (MTS). Data were entered at an IBM 2741 typewriter console in response to prompting from ALGOL W and FORTRAN programs, and the resultant graphical output appeared on a Computek 400 visual display screen connected to the IBM 360 via a PDP 11. Hard copy graphs were obtained using a Benson-Lehner graph plotter.

Output from the computer models

Six runs of model I are shown in figure 1. The graphs are essentially the same as those given by Kimura ('70), but it must be emphasized that in a teaching situation the parameters for successive runs may be decided upon by the teacher in order to illustrate any feature which is causing difficulty, or they may be chosen by the students. If, for instance, students find the concept of the rate of fixation difficult to grasp, successive runs in which only the number of generations is altered may be carried out. When the process seems to have been understood an invitation to predict the probability of fixation at a point in a similar sequence with other values of population size or initial frequency may be extended.

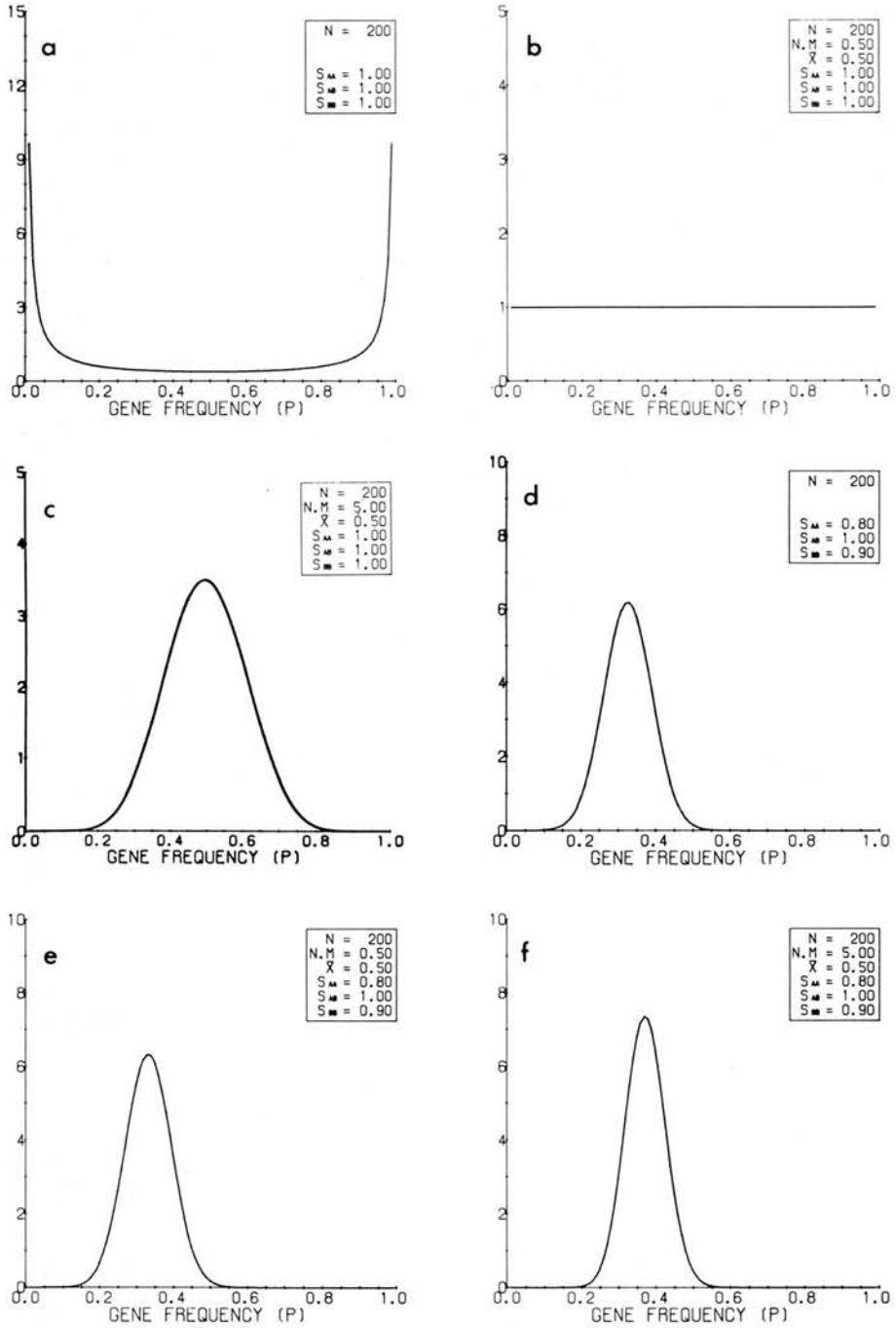
It is important that each graph should be scrupulously annotated to prevent misunderstandings due to differences in scale or confusion between models. Points which may be brought out clearly include the dependence of the gene frequency distribution upon t (number of generations) and N (effective size) only through their quotient, i.e., the gene frequency distribution in populations of effective size 200 after 50 generations is the same as that in populations of effective size 100 after 25 generations. Also important are the increasing probability of fixation with time, and with the departure of the initial gene frequency (p) from 0.5, and the inevitability of final fixation.

Figure 2 shows the results of six runs of model II which illustrate various effects of selection and migration. S represents the fitness of the genotype shown in the suffix, $\bar{x}$ is the immigrant gene frequency and N.M is the product of the effective size and the migration rate. Figures 2a,b,c show



GENE FREQUENCY DISTRIBUTION FOR UNFIXED POPULATIONS AT A DIALLELIC LOCUS UNDER RANDOM GENETIC DRIFT

Fig. 1 Sample output from model I. The program is given the population size, initial gene frequency and number of generations of drift, and it produces the graph and the probability of fixation. The scale of the vertical axis is such that the area under the curve is the proportion of unfixed populations.



GENE FREQUENCY DISTRIBUTION FOR A DIALLELIC LOCUS IN EQUILIBRIUM UNDER SELECTION AND MIGRATION

Fig. 2 Sample output from model II. The program is given the population size (N), the migration rate (M) the gene frequency ($\bar{x}$) of the immigrants, and the fitnesses of the genotypes, and it produces the graph. The scale of the vertical axis is such that the area under the curve is unity. N.M. is the number of immigrants per generation. In figures a and d, $M = 0$.

the effect of varying migration rates, and comparison with d, e, f the effect of different fitnesses. Again, teachers and students should work out between them just which values of the parameters must be considered next so as to make a specific point. In the course of a one-hour session it would be quite feasible to draw some 20 such graphs, from which a great deal of information can be derived. The effects of lethal recessives and gametic selection can be discussed as well as the effects of superior heterozygotes, but it must be remembered that the model caters for those populations in which both genes are present. It may be thought instructive to show that the formula given by Li ('55), which seems to indicate that selection is acting only through the product of the population size and the heterozygote advantage, is an approximation which should not be accepted as a general result.

Figure 3 shows four runs of model III applied to a triallelic locus. The topics which can be covered using this model include the effect of population size and migration rate as with two genes. Furthermore, conditions on the genotype fitnesses for the stability of an equilibrium situation, which now become more involved, can be investigated. It is in the triallelic case that the use of such a model in an interactive context has the greatest advantage over other media in giving students insight into the system. In the simpler case of a diallelic locus the use of the technique described is con-

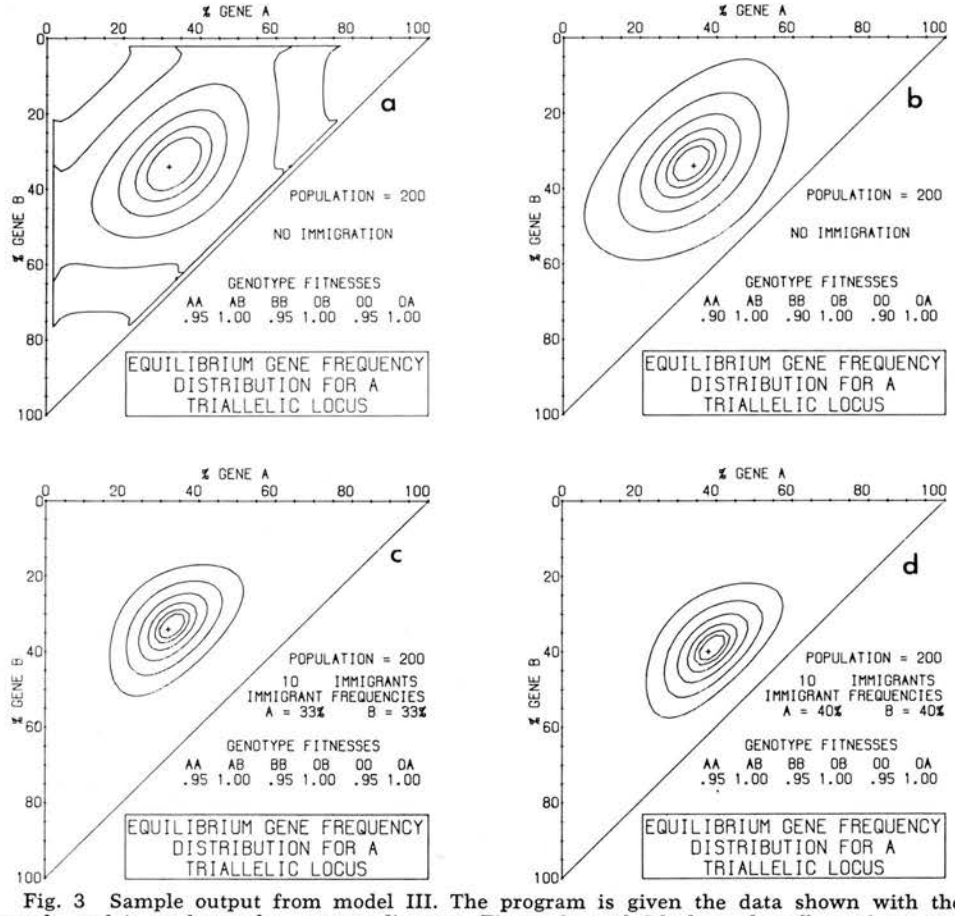
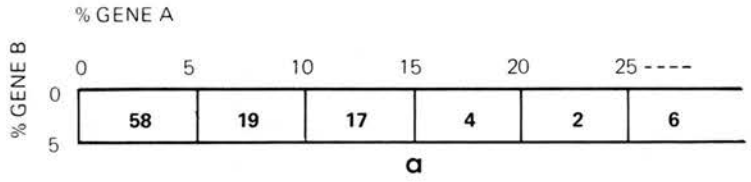


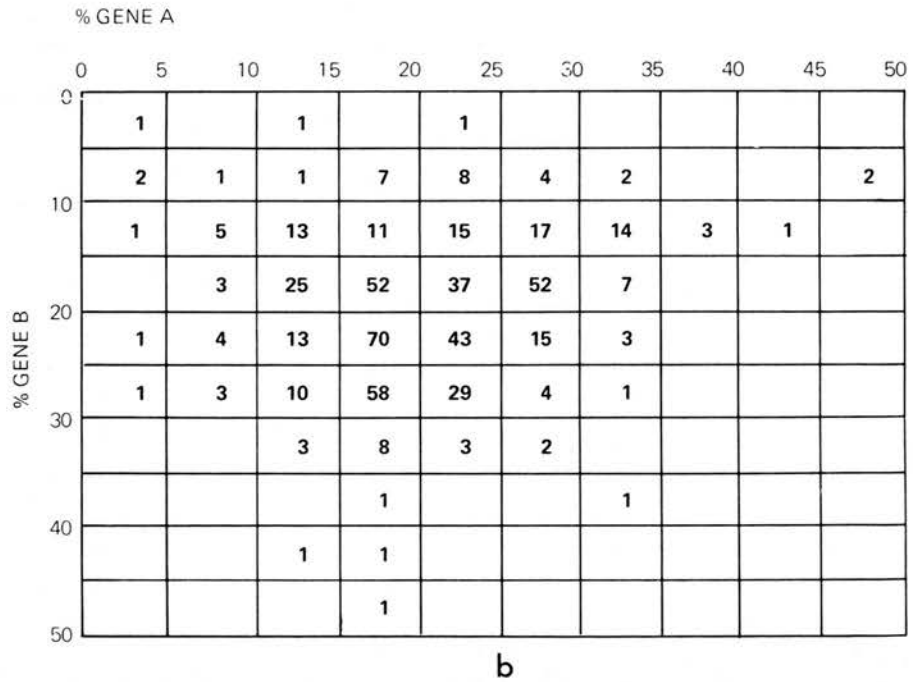
Fig. 3 Sample output from model III. The program is given the data shown with the graph, and it produces the contour diagram. Figure 3c and 3d show the effect of ten immigrants per generation.

venient and useful, but it cannot be denied that the facts can be adequately taught without such a method. To understand the various ramifications of an alteration in parameters in the triallelic case, however, a quick method of producing intelligible results is imperative. Figure 3a and 3b show clearly the effect of slight variation in fitnesses and comparing them with c and d shows the restraining effect on the gene frequency distribution of 5% immigration per generation.

Numerical output as illustrated in figure 4 could have been given, but although on occasion such a table is useful it does not give an easily comprehensible overall impression of the situation, particularly in the triallelic situation of figure 4b. Alternatively an attempt could have been made to draw a three dimensional representation of the probability distribution, but few satisfactory, informative, unambiguous diagrams can be produced quickly. A contour diagram is a convenient compromise between these two extremes. Percentile contours could have been drawn, but this would have entailed calculation which would have slowed down the response of the program unacceptably in the teaching situation. Because the expression for the frequency distribution involves the term $\bar{w}^{2N}$, small changes in the mean fitness, due to small departures from the equilibrium position, can cause large changes in the value of the probability density function. The contours drawn were not therefore



a



b

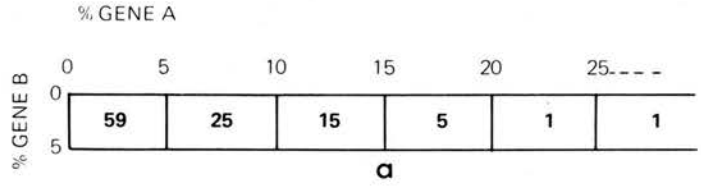
Fig. 4 Gene frequency distributions calculated from observed phenotype frequencies by Bernstein's method for figure 4a, 106 American Indian samples (low frequency of B gene gives a virtually diallelic system); and figure 4b, 562 Asian samples (triallelic).

equally spaced heights but were chosen so that the representation of the distribution was more meaningful to the eye. The outer contour of the six drawn represents a very low probability indeed.

Although the model for genetic drift for a triallelic locus over a given number of generations could be programmed as well as the equilibrium situation of model III, it is felt that this would not assist very much the students' understanding after they had seen the other models.

Validity of the models

Mathematical models of systems often arouse suspicions in students (and others) that the simplifications necessary to make the model tractable render it so unlike the actuality as to be worthless. It is therefore important that the distributions produced by the models should be demonstrated to bear a reasonable resemblance to those found by observation. Nassar ('69) has investigated the agreement between model I and a Monte-Carlo model and found it to be good even for quite small populations. For models II and III the ABO locus is suitable for consideration, firstly because large quantities of data are available, secondly because there is good evidence from experimental studies that selection is acting, and thirdly because it fulfills the need for



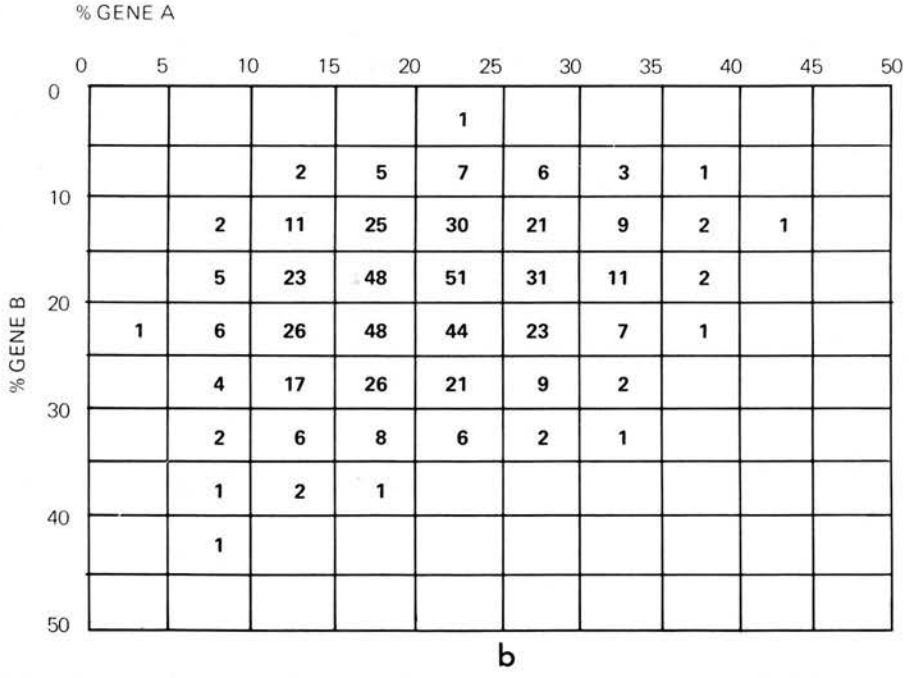


Fig. 5 Gene frequency distributions from the models.

Fig. 5a One hundred and six populations of size 200 with fitnesses of AA, AO and OO equal to 0.75, 1.00 and 0.97 with no migration.

Fig. 5b Five hundred and sixty-two populations of size 200, five immigrants per generation from a population with 20% gene A and 20% gene B. Genotype fitnesses for AA, AB, BB, OB, OO and OA are 0.91, 1.06, 0.90, 1.05, 1.00 and 1.05.

a triallelic locus in most parts of the world and will double as a diallelic locus if the American Indians are examined.

Because such parameters as migration rate and population size are not known sufficiently accurately for the populations for which we have data we cannot legitimately use our models to estimate selective intensity for the different genotypes. It is nevertheless informative and important to relate the models to observations.

Bernstein's ('30) method was used to estimate the frequencies of genes A, B and O from the phenotype frequencies given by Mourant, Kopec and Domaniewska-Sobczak ('58) for several geographical regions. This is not entirely satisfactory as Bernstein's method assumes Hardy-Weinberg equilibrium which will not strictly be the case if selection and migration are in operation, but it should suffice for present purposes. (It is interesting to note that Bernstein obtained the correct result after making an unjustified approximation to overcome a mathematical error.) The groups considered here are the American Indians (106 samples) and populations from Asia (562 samples). The gene frequency distributions calculated from these samples are shown in figures 4a and 4b respectively. Figure 5 shows expected frequency distributions for the parameters specified. There is a greater scatter in the observed figures but the agreement is close enough to indicate that the models used are not unreasonable. No attempt was made to fit the data more closely, simplicity being considered more important than precision. Taylor ('71) has pointed out the different requirements in terms of accuracy and simplicity of simulation programs which are to be used in teaching compared with those which are to be used in research. He is in fact discussing analog and hybrid methods, but his observations are relevant here as the use of fast graphical methods with a digital computer has many similarities to the hybrid situation. In the present instance it is necessary for a few important ideas to be communicated to students with as much clarity and as little unnecessary complexity as possible.

Use of the models

The models were used to teach a class of ten third year honours genetics (undergraduate) students at the University of Newcastle upon Tyne. Interest shown by the students was considerable; they were keen to provide parameters for each successive graph, and indeed the lesson tended to proceed rather too quickly for the efficient retention of information, a defect which will easily be rectified in future years. The ability of migration alone to counteract the effect of drift surprised the class, and the fact that they discovered for themselves that this same effect was of second order importance in the presence of a stable polymorphism should leave a lasting impression of the dramatic effect of selection. As well as the output produced on the display screen hard copy output was available for several values of the parameters. This is necessary if a useful summary of the lesson is to be given afterwards.

It is appreciated that many institutions whose students would benefit from the use of interactive computer models as described here may lack the necessary graph plotting facilities. Some progress in this direction may be made by utilising a standard line printer; the same convenience is lacking, and organisational difficulties may tend to obscure the main object of the lesson, but useful results could be obtained. For example, contour diagrams which bring out the salient features of the distributions for the triallelic locus may be output on a line-printer.

APPENDIX

THE STEADY STATE GENE FREQUENCIES FOR A MULTIALLELIC LOCUS UNDER SELECTION AND MIGRATION

The diffusion equation approach to gene frequency distributions

The most general form of the Fokker-Planck equation is:

$$\frac{\partial \phi}{\partial t} = \tfrac{1}{2} \sum_{j=1}^{k-1} \frac{\partial^2}{\partial x_j^2} \{ V(\delta x_j)\phi \} + \tfrac{1}{2} \sum_{\substack{i=1 \\ i \neq j}}^{k-1} \sum_{j=1}^{k-1} \frac{\partial^2}{\partial x_i \, \partial x_j} \{ W(\delta x_i, \delta x_j)\phi \} - \sum_{j=1}^{k-1} \frac{\partial}{\partial x_j} \{ M(\delta x_j)\phi \} \quad \ldots (1)$$

where the notation used is as follows:

$\phi(x_1, x_2, \ldots, x_k; t)$ is the joint probability density function, t generations after some initial state, for the gene frequencies x_i of the k alleles A_i at an autosomal locus; x_1, $x_2, \ldots, x_{k-1}$ are taken as the independent variables, with $x_k = 1 - \sum_{i=1}^{k-1} x_i$.

δx_j is the change in x_j per generation;

$M(\delta x_i)$ is the expected value of δx_i;

$V(\delta x_i)$ is the variance of δx_i;

$W(\delta x_i, \delta x_j)$ is the covariance of δx_i and δx_j.

The equation only deals with the case in which all k alleles are present, i.e., $x_i \neq 0$, $1 \leq i \leq k$.

Kimura ('64) has introduced the valuable idea of "probability flux"; we shall call the probability flux along the x_i axis $P(x_i)$ and write

$$P(x_i) = \tfrac{1}{2} \frac{\partial}{\partial x_i} \{ V(\delta x_i)\phi \} + \tfrac{1}{2} \sum_{\substack{j=1 \\ j \neq i}}^{k-1} \frac{\partial}{\partial x_j} \{ W(\delta x_i, \delta x_j)\phi \} - M(\delta x_i)\phi \quad \ldots (2)$$

For a steady state solution $\frac{\partial \phi}{\partial t} = 0$ and the equation to be solved is therefore

$$\sum_{i=1}^{k-1} \frac{\partial}{\partial x_i} P(x_i) = 0.$$

The solution which is of interest is the "zero flux" equilibrium solution which, if it exists, is given by $P(x_i) = 0$ for $1 \leq i \leq k-1$. $\ldots (3)$

Selection and migration

Suppose we have k alleles A_i at an autosomal locus, $1 < i < k$. If the frequency of A_i is x_i and the fitness of genotype A_iA_j is s_{ij}, then we can write the mean fitness $\overline{w} = \sum_{i=1}^{k} \sum_{j=1}^{k} s_{ij}x_ix_j$. If ${}^sM(\delta x_i)$ is the expected change in the frequency of A_i due to selection, then ${}^sM(\delta x_i) = \frac{1}{\overline{w}} \sum_{j=1}^{k} s_{ij}x_ix_j - x_i$.

Suppose that there is migration also, and that our population exchanges members with a random sample of the total population at a rate m per head of population per generation. Let x_i be the frequency of A_i in the total population, and write ${}^mM(\delta x_i)$ as the expected change in x_i due to migration. Then ${}^mM(\delta x_i) = m(\overline{x_i} - x_i)$. We can write $M(\delta x_i) = {}^sM(\delta x_i) + {}^mM(\delta x_i)$.

If the variance of δx_i arises only because of random sampling of gametes we have, for a population of effective size N:

$$V(\delta x_i) = \frac{1}{2N} x_i(1 - x_i)$$

$$\text{and } W(\delta x_i, \delta x_j) = \frac{-1}{2N} x_ix_j$$

We may rewrite equations (2) and (3) as

$$\frac{\partial}{\partial x_i} \{ x_i(1 - x_i)\phi \} - x_i \sum_{\substack{j=1 \\ j \neq i}}^{k-1} \frac{\partial}{\partial x_j} \{ x_i\phi \} = \{ \frac{4Nx_i}{\overline{w}} \sum_{j=1}^{k} s_{ij}x_j - 4Nx_i + 4Nm(\overline{x_i} - x_i) \} \phi$$

which reduces to

$$x_i(1 - x_i) \frac{\partial \phi}{\partial x_i} - x_i \sum_{\substack{j=1 \\ j \neq i}}^{k-1} x_j \frac{\partial \phi}{\partial x_j} = \{ \frac{4Nx_i}{\overline{w}} \sum_{j=1}^{k} s_{ij}x_j - 4Nx_i + kx_i + 4Nm(\overline{x_i} - x_i) - 1 \} \phi \quad \ldots (4)$$

Solution of the equations

In order to solve equations (4) we consider the matrix H defined by

$$H_{ij} = -x_i x_j,\ i \neq j;\ H_{jj} = x_j(1 - x_j).$$

H is proportional to the variance-covariance matrix of δx_i and δx_j. It is a symmetric $(k-1) \times (k-1)$ non-singular matrix, having determinant $\prod_{i=1}^{k} x_i$. We may find its inverse which we shall call K.

$$K_{ij} = \frac{1}{x_k},\ i \neq j;\ K_{jj} = \frac{1}{x_k} + \frac{1}{x_j}.$$

Since equation (4) is equivalent to

$$H\frac{\partial \phi}{\partial \underline{x}} = \phi \underline{f}$$

where $\frac{\partial \phi}{\partial \underline{x}} = \left(\frac{\partial \phi}{\partial x_1}, \frac{\partial \phi}{\partial x_2}, \ldots, \frac{\partial \phi}{\partial x_{k-1}}\right)'$,

and f is a $(k-1)$ vector with

$$f_i = \frac{4Nx_i}{\bar{w}} \sum_{r=1}^{k} s_{ir}x_r + kx_i - 4Nx_i + 4Nm(\bar{x}_i - x_i) - 1,$$

it follows that $\frac{\partial \phi}{\partial \underline{x}} = \phi K \underline{f}$ . . . (5).

$$\text{Now } (K\underline{f})_j = \sum_{i=1}^{k-1} K_{ij}f_i = \frac{1}{x_k}\sum_{i=1}^{k-1} f_i + \frac{f_j}{x_j}$$

$$= \frac{1}{x_k}\frac{4N}{\bar{w}}\{\bar{w} - x_k \sum_{r=1}^{k} s_{kr}x_r\}$$

$$+ \frac{1}{x_k}\{(k - 4N - 4Nm)(1 - x_k) + 4Nm(1 - x_k) + 1 - k\}$$

$$+ \frac{4N}{\bar{w}} \sum_{r=1}^{k} s_{jr}x_r + (k - 4N - 4Nm) + \frac{4Nm\bar{x}_j - 1}{x_j}$$

$$= \frac{4N}{w} \sum_{r=1}^{k} (s_{jr} - s_{kr})x_r + \frac{1 - 4Nm\bar{x}_k}{x_k} - \frac{1 - 4Nm\bar{x}_j}{x_j}$$

Furthermore $\sum_{r=1}^{k} (s_{jr} - s_{kr})x_r = \tfrac{1}{2}\frac{\partial \bar{w}}{\partial x_j}$, and,

writing $\alpha_j = 1 - 4Nm\bar{x}_j$ for convenience leads to $(K\underline{f})_j = 2N\frac{\partial}{\partial x_j}(\log \bar{w}) + \frac{\alpha_k}{x_k} - \frac{\alpha_j}{x_j}$.

Therefore equation j from the system (5) is equivalent to

$$\frac{\partial \phi}{\partial x_j} = \left\{2N\frac{\partial}{\partial x_j}(\log \bar{w}) + \frac{\alpha_k}{x_k} - \frac{\alpha_j}{x_j}\right\}\phi \quad . . . (6).$$

If $G_j(\underline{x})$ is an arbitrary function of $x_1, x_2, \ldots, x_{k-1}$ but does not involve x_j, then integration gives

$$\log \phi = \log \bar{w}^{2N} - \log x_k^{\alpha k} - \log x_j^{\alpha j} + \log G_j(\underline{x})$$

$$\text{i.e., } \phi = G_j(\underline{x})\frac{\bar{w}^{2N}}{x_k^{\alpha_k} x_j^{\alpha_j}}$$

Since this must hold for $j = 1, 2, \ldots, k-1$ the only possible solution is $\phi = \frac{C\bar{w}^{2N}}{\prod_{i=1}^{k} x_i^{\alpha_i}}$ where C is chosen to make $\int_R \phi(\underline{x})\, d\underline{x} = 1$ for the region R:

$0 < x_i < 1$, $(1 \leq i \leq k-1)$, and

$0 < \sum_{i=1}^{k-1} x_i < 1$.

Notice that equation (6) leads directly to the conclusion that modal equilibrium gene frequencies for a large population are given by $\phi\frac{\partial}{\partial x_j}(\log \bar{w}) = 0$, or more simply, $\frac{\partial \bar{w}}{\partial x_j} = 0$, $j = 1, 2, \ldots, k-1$; which is the condition for the average fitness to be a maximum.

CONCLUSION

We have proved that the diffusion equation leads to the result that, for a locus with k alleles A_i with frequency x_i and mean fitness $\bar{w}$, in a population of effective size N subject to migration at rate m per generation from a population in which A_i has frequency $\bar{x}_i$, the steady state gene frequency distribution is given by $\phi(x_1, x_2, \ldots, x_k) = \frac{C\bar{w}^{2N}}{\prod_{i=1}^{k} x_i^{1-4Nm\bar{x}_i}}$. This is a straightforward extension of the well known result for a diallelic locus.

ACKNOWLEDGMENTS

I am indebted to Dr. D. F. Roberts not only for using the system with his students, but also for his encouragement and helpful discussions on many topics relevant to this communication. I also wish to thank my Head of Department Professor D. J. Newell and my Supervisor Professor E. S. Page for their advice; and Dr. G. Shearing for writing the Computek software.

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GENE FREQUENCIES FROM FAMILY DATA

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Introduction

Studies of gene frequencies in populations, whether in relation to their geographical location or to a particular disease group, are usually based on data from random samples of the population under investigation. Many genetics laboratories, however, in Britain and elsewhere possess considerable quantities of information in the form of family data, and this can be utilized to give estimates of gene frequencies and to make comparisons between two or more groups. This may be particularly relevant when the groups are of limited size, as for instance in the case of a rare disease. A method by which this can be done is set out in this paper.

Method

Li (1955) gives a maximum-likelihood (ML) method of estimating gene frequencies using mother–child (or more generally parent–offspring) pairs for the case of 2 alleles without dominance at an autosomal locus. The method is based on the assumptions of random mating and Hardy-Weinberg equilibrium. Such systems as MN, Ss, AK, Hp and PGM may be investigated using this method.

Table 1 shows the genotype frequencies for offspring of all matings, given the above conditions, and Table 2 shows the same frequencies condensed by mother's genotype. p represents the (fractional)

frequency of gene A, and the frequency of a is $q = 1 - p$. If data are collected and arranged in a form equivalent to that of Table 2, Table 3 results; the n_i are observed numbers. If $n = \sum_{i=1}^{7} n_i$ is the total number of mother–child combinations there are 2n individuals with 4n genes. The gene frequencies may be estimated from the n mothers alone or from the n children, but because each child must share a

TABLE 1. Frequency of genotype of offspring for all possible matings in a random mating population with a 2 gene system in Hardy-Weinberg equilibrium

Mating ♀	Mating ♂	Frequency of mating	Genotype of offspring AA	Aa	aa
AA	AA	p^4	p^4		
AA	Aa	$2p^3q$	p^3q	p^3q	
AA	aa	p^2q^2		p^2q^2	
Aa	AA	$2p^3q$	p^3q	p^3q	
Aa	Aa	$4p^2q^2$	p^2q^2	$2p^2q^2$	p^2q^2
Aa	aa	$2pq^3$		pq^3	pq^3
aa	AA	p^2q^2		p^2q^2	
aa	Aa	$2pq^3$		pq^3	pq^3
aa	aa	q^4			q^4

gene with its mother there are only 3n independent genes, and it is not permissible to pool the mothers and children to provide an estimate using both. It is however desirable to use all the data available and the correct procedure is to apply the maximum-likelihood technique to the 7 cells of the table. The log-likelihood L is given by

$$L = n_1 \log p^3 + (n_2 + n_3) \log p^2q + n_4 \log pq + (n_5 + n_6) \log pq^2 + n_7 \log q^3.$$

Collecting terms,

$$L = B \log p + C \log q$$

where

$$B = 3n_1 + 2(n_2 + n_3) + n_4 + (n_5 + n_6)$$

and

$$C = (n_2 + n_3) + n_4 + 2(n_5 + n_6) + 3n_7.$$

The maximum-likelihood equation is $\frac{dL}{dp}=0$

and, remembering that $q=1-p$, it may be solved for p, giving

$$p=\frac{B}{B+C}.$$

TABLE 2. Frequency of genotype of offspring for all possible maternal genotypes in a random mating population with a 2 gene system in Hardy-Weinberg equilibrium.

		Child		
		AA	Aa	aa
Mother	AA	p^3	p^2q	
	Aa	p^2q	pq	pq^2
	aa		pq^2	q^3

TABLE 3. Observed frequencies of mother-child genotype combinations.

		Child		
		AA	Aa	aa
Mother	AA	n_1	n_2	
	Aa	n_3	n_4	n_5
	aa		n_6	n_7

The ML equations may be derived in a similar fashion for any number of alleles, with or without dominance. In the case of 2 genes with dominance the ML equation for the gene frequency estimate may be reduced to a cubic. For more than 2 alleles the equations may be solved using matrix algebra. An iterative method of solution is used, which employs the information matrix M given by

$$M_{ij}=-\frac{\partial^2 L}{\partial p_i \partial p_j}.$$

Starting from an approximation $\mathbf{p}^{(0)}$ to the set of gene frequencies $\mathbf{p}=(p_1, p_2, \ldots p_{k-1})$, for a k-allelic locus a better approximation

$\mathbf{p}^{(1)}$ is found by

$$\mathbf{p}^{(1)} = \mathbf{p}^{(0)} + \left[\mathrm{M}^{-1} \frac{\partial \mathrm{L}}{\partial \boldsymbol{p}} \right]_{\boldsymbol{p}^{(0)}}$$

The process is repeated until 2 successive approximations agree sufficiently closely. The variance–covariance matrix for the gene-frequency estimates and the expected numbers of each mother–child combination are readily found, in addition to the gene-frequency estimates themselves.

A computer program which performs the analysis for any of the 2 or 3 gene cases has been written (in APL for use on an IBM 360/67) and used to provide the results which follow.

Results

To illustrate the usefulness of the method preliminary data for blood groups and isoenzymes from a series of families in which juvenile peptic ulcer is present were examined by the method outlined above, and the gene frequencies were compared with a series of control families. The material was made available by courtesy of our colleagues Dr. R. H. Jackson and Dr. W. M. Cowan.

The MN groups were treated as a 2-gene system without dominance; the Rhesus (D) positive and negative as a 2-gene system with dominance; the acid phosphatase types as a 3-gene system without dominance; and the ABO blood groups as a 3-gene system with dominance. Table 4 gives a summary of the results obtained. The deficiency of ABO gene A in the peptic ulcer series is significant at the 5 per cent level.

Table 5 gives a summary of the results obtained from an analysis of the children's data alone. No significant differences were detected between the two series. In no case was there a significant difference between the gene frequencies estimated from the children and those estimated from the mother–child pairs, but the smaller standard errors (reduced by a factor of about 0·8)which result from using the larger sample provide the method with its additional power. This is achieved at little extra cost as the use of mother–child pairs is a most convenient way of increasing the size of a sample.

Since the maximum-likelihood method used for gene-frequency estimation depends on the assumption of Hardy–Weinberg equilibrium, the method of Smith (1970) was used when dominance was

TABLE 4. Gene frequency estimates for peptic ulcer and control populations using mother-child data for 4 genetic system (2 and 3 genes with and without dominance); with sample sizes, standard errors and the probabilities that observed differences are due to chance.

Gene		Disease group			Controls			Difference	
		Sample size	Gene frequency	Standard error	Sample size	Gene frequency	Standard error	Normal deviate	Probability
ABO	A	48	0·172	0·033	47	0·277	0·042	1·97	$p<0·05$
	B	48	0·081	0·023	47	0·051	0·019	1·01	n.s.
	O	48	0·747	0·038	47	0·672	0·043	1·31	n.s.
Rh	(D)	48	0·605	0·053	47	0·538	0·052	0·90	n.s.
MN		48	0·537	0·043	47	0·444	0·046	1·48	n.s.
AcPh	A	33	0·392	0·051	32	0·263	0·047	1·86	n.s.
	B	33	0·566	0·052	32	0·672	0·050	1·47	n.s.
	C	33	0·042	0·021	32	0·065	0·026	0·69	n.s.

TABLE 5. Gene frequency estimates for peptic ulcer and control populations using data for children only for 4 genetic system (2 and 3 genes with and without dominance); with sample sizes, standard errors and the probabilities that observed differences are due to chance.

Gene		Disease group			Controls			Difference	
		Sample size	Gene frequency	Standard error	Sample size	Gene frequency	Standard error	Normal deviate	Probability
ABO	A	48	0·183	0·042	47	0·272	0·050	1·36	
	B	48	0·075	0·027	47	0·055	0·024	0·55	
	O	48	0·742	0·047	47	0·674	0·052	0·97	not
Rh	(D)	48	0·592	0·066	47	0·516	0·064	0·83	significant
MN		48	0·552	0·051	47	0·468	0·051	1·16	
AcPh	A	33	0·409	0·061	32	0·266	0·055	1·74	
	B	33	0·530	0·061	32	0·656	0·059	1·48	
	C	33	0·061	0·029	32	0·078	0·034	0·38	

absent to test for significant deviations from that situation. None were found.

Summary

The use of mother–child pairs provides a sensitive method for estimating gene frequencies and is particularly useful where data are limited. The method is illustrated by a comparison of isoenzyme gene frequencies between a series of families with juvenile peptic ulcer and a control series.

Acknowledgements

Thanks are due to Dr. R. H. Jackson and Dr. W. M. Cowan for making available the data on which this study is based, and to Dr. D. F. Roberts who suggested this approach.

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Appendix

The authors will be happy to process any parent–offspring data sent to them. The computer program requires data in one of the following forms.

I. 2 genes without dominance.

		Child		
		AA	Aa	aa
Mother	AA	x	x	
	Aa	x	x	x
	aa		x	x

II. 2 genes with dominance.

		Child	
		Dom	Rec
Mother	Dom	x	x
	Rec	x	x

III. 3 genes without dominance.

		Child					
		CC	CA	AA	CB	BB	AB
Mother	CC	x	x		x		
	CA	x	x	x	x		x
	AA		x	x			x
	CB	x	x		x	x	x
	BB				x	x	x
	AB		x	x	x	x	x

IV. 3 genes with dominance.

		Child			
		O	A	B	AB
Mother	O	x	x	x	
	A	x	x	x	x
	B	x	x	x	x
	AB		x	x	x

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Human Nutrition: Applied Nutrition (1983) 37A, 293-300.

USE OF A DIETARY DIARY AND INTERVIEW TO ESTIMATE THE FOOD INTAKE OF CHILDREN

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A recently completed large-scale longitudinal dietary survey is described. The diary and interview method was used within the normal school organisation and proved to be a very acceptable and efficient means of collecting dietary data. Very few problems were encountered with the technique and a relatively low volunteer rate was compensated for by a very low drop-out rate. Useful quantitative dietary data is thought to have been obtained and this will be reported at a later date. The data required were primarily concerned with frequency of eating and macronutrient intake; the diary and interview method may be less acceptable for other uses and with other age groups.

Introduction

The measurement of voluntary food intake is probably one of the most difficult of physiological measurements (Garrow, 1974). However, the problems arise less from recording food intake than from acquiring a representative sample of the population, studying an adequately large sample, converting food intakes into nutrient intakes and obtaining a good estimate of an individual's average intake. The measurement of food intake has been reviewed many times, for example by Marr (1971). However, the aim of this paper is to describe a recently completed dietary survey whose salient features may warrant greater usage.

Aims of the survey

The main aim of the survey was to determine which dietary factors had the greatest influence (positively or negatively) on the development of dental caries in adolescent schoolchildren. Since the development of caries is slow, the study had to last at least two years to allow an adequately large number of new carious lesions to develop; the dietary intake was assessed over the same two-year period. By using multivariate analysis the dietary factors ('independent' variable) can be ranked in the order in which they correlate, independently of the others, with the observed caries increment (the 'dependent' variable). This information can then be used to modify or reinforce current dietary advice concerning dental health.

Choice of method

There were the following constraints on the survey: (1) the subjects were initially 11-years old (the age when a large caries increment may be expected) and of mixed ability and background (to provide varied eating habits); (2) average intake over two years was required for each individual; (3) because of the

multiplicity of factors known to influence the occurrence of caries, and the large number of possible sources of error, a large sample size was required and it was therefore decided to obtain complete records from at least 400 individuals for final statistical analysis; (4) each of the variables had to be estimated with high reliability for each child; (5) only one nutritionist was available to carry out the dietary survey; (6) there were no facilities for food analysis; (7) a six-month planning period was allowed in the protocol for the collection of essential background data; (8) although the current view is that the frequency of intake of sugars is of primary importance, there have been suggestions that other dietary factors such as fat, calcium and phosphorus may be important (Jenkins, 1978) and therefore a wide variety of dietary factors were investigated; (9) in order to ensure a high two-year caries increment it was necessary to travel outside the fluoridated Newcastle area, thus greatly increasing the travelling time.

Weighing of intake was impractical because of the lack of resources for adequate supervision and home visits. On the other hand, several studies have investigated the accuracy with which children can recall their food intake (eg, Bransby, Daubney & King, 1948; Meredith *et al.*, 1951; Emmons & Hayes, 1973; & Frank *et al.*, 1977), and on the basis of their reports it was decided that dietary histories would not be adequate for our purposes. A diary record in household measures was therefore the method of choice. Records for five or seven days were expected to be less popular with the children than shorter records and, in any case, the reliability is little improved by increasing the number of days from three to five (Chalmers *et al.*, 1952; Gardner & Heady, 1973). A three-day dietary diary is cheaper (in stationery, interviewing and processing time) than a five- or seven-day diary, and if replicated five times would probably be as good as a five-day survey replicated four times. An interview with each child on the fourth day to clarify doubtful entries and quantify the data was found to be essential, and this procedure of three-day diary plus interview was repeated on five occasions during the two-year period to allow for variants such as the day of the week, individual variation, growth and ageing, season, holidays, illness and other special occasions. (Each individual's dietary collections were arranged to ensure as far as possible, an unbiased sampling of weekdays). Furthermore, the order in which the schools were visited was altered to ensure that records were kept during all seasons of the year and when possible records were kept during school holidays. The days on which the records were kept were decided by: the inability to interview on weekends or school holidays, the undesirability of interviewing more than 15 children on one day, the days or seasons during which the previous record had been kept and the desire to interview children from only one school on a given day (apart from absentees and/or defaulters). A total of 15 days' intake per child was recorded. All data collection was undertaken in the children's schools, which greatly simplified the organisation.

Organisation of the study

Permission for the study was obtained from the Northumberland Area Health Authority and County Education Authority, and from headteachers of seven of the eight middle schools in the Morpeth, Newbiggin and Ashington area of

Northumberland. In July 1979 all second-year middle-school children (average age 11½ years) at these seven schools were invited to volunteer for the study. A letter was sent to each child's parents describing the study in detail and written parental permission was requested; the headteachers also described the study to the children. Four hundred and seventy children volunteered, corresponding to 59 per cent of the schools' rolls. This rather low figure could probably have been improved if the investigators had talked to the children initially to make the study more special and exciting to them (Thomson, 1958; Marr, 1971). However, the low acceptance rate probably helped to achieve an extremely low drop-out rate (only two children asked to leave the study during the two years). It is possible that the sample was not representative of those who declined to participate but this was not investigated. Both sexes were almost equally represented in the final sample of 405 children who completed all aspects of the study (193 boys, 212 girls), and social classes (I 20, II 65, III 172, IV 96, V 22, others 30) were represented in proportions similar to those expected in south Northumberland as a whole.

In September/October 1979 the volunteers at each school were instructed *en masse,* using visual aids, on how to complete the dietary records. Professor G.N. Jenkins also outlined the study to the children, and made them feel important. They asked many questions and every effort was made to emphasise the universal importance of this project and to generate enthusiasm. Particular care was taken to divorce as far as possible the dental and dietary aspects of the study. To investigate the possibility of biased recording because of association with the dental examinations, similar volunteers were recruited from the eighth school in this area and told nothing of the dental aspects of the survey. The children were from similar backgrounds to the children in the main study and kept one diary for three days and were not given dental examinations. The results of this subsidiary study will be published in due course.

Each child was provided with a new pocket-sized (148 x 105 mm) diary for each survey as they were expected to carry the diaries with them for the three days. The diaries also needed to be fairly robust; they cost approximately 20 p each. Each diary contained instructions, an example of its use, and eight pages of 26 lines for recording the food consumed. Each of these pages was divided into three columns for recording the time of intake, what was consumed and an estimate of how much. At the top of each page there was provision for identifying the subject, the survey and the date of the record. If possible the diaries were distributed the day before recording began, with a 'pep' talk from the investigator. The children were then left to record their diets and were not seen again until the interview on the fourth day.

It was essential that the interviews were conducted on the fourth day while the children's memories were fresh. The interviews had to take place at school and, to allow time to process the diaries, it was preferable to interview only during the mornings, the diaries were therefore distributed to groups of 10-15 children at one time. During the first survey, in 269 attempted interviews, absentees amounted to 9 per cent and lost or forgotten diaries to 2 per cent and 18 per cent respectively. Each interview was found to last 19 ± 4 min (mean ± s.d.), and this usually led to a full morning's work per group, although absentees

and children with lost or forgotten diaries had to be added to the work load on succeeding days.

At the interview the dietitian worked systematically through each child's diary. Vague descriptions were clarified, any gaps or other unusual events probed, and four pieces of information were obtained for each food item recorded. These were the time of the intake, what was eaten, its quantity in household measures and whether it was eaten with any other item. The child's bedtime was also recorded. A gram weight for each item was arrived at using the child's description in household measures of each food item, with the help of a selection of calibrated utensils such as cups, spoons, glasses and a variety of polystyrene shapes of known volume. This equipment undoubtedly speeded the interview and probably introduced some degree of objectivity. Standard weights for each food volume were assigned: this method has been shown to be useful in the past (Moore, Judlin & Jennemur, 1967). These weights, any other information, and later McCance and Widdowson's (MW4) food codes (Paul & Southgate, 1978), were written into the diaries by the interviewer. Finally the interview was used to maintain enthusiasm for the remainder of the survey. It was obvious as time went on that each child became more expert at keeping the diaries: the interviews shortened slightly and information was more easily provided by the children. A rapport gradually developed which was carefully cultivated, for example a film show was given at the end of the first year, and cards were sent each Christmas to each child. There was a steady flow of extra volunteers.

The number of diaries collected is shown in Table 1. During surveys I and II, interviews were conducted on three or four days per week, but during surveys III, IV and V these increased to four or five days per week. This accounts for the increased number of diaries collected in the second year. Seventeen diaries from five subjects were rejected as being too poorly kept to be of use. There were a number of other doubtful diaries usually claiming exemplary eating habits; these were all vigorously probed at the interview, but unless the grounds for rejecting them were irrefutable they were allowed to remain in the study. A child was only excluded from the study if more than two of his or her diaries were classifed as rejects. All 15 days were recorded satisfactorily by 405 children (86 per cent of the total number of volunteers).

The next phase of the survey consisted of allocating a MW4 food code to each item recorded. This took an average of 7 ± 3 min (mean ± s.d.) per diary, not including the time spent coding new non-MW4 items. All the diary information was then transcribed onto coding sheets to allow computer punch cards to be

Table 1. *Number of diaries (one diary per subject) collected at each of the five dietary surveys conducted over a 23-month period.* The aim was for at least 400 subjects to complete all five surveys and dental examinations; 405 did so.

	Survey					
	I	II	III	IV	V	Total
Number of diaries	466	461	448	451	443	2269
Date (inclusive)	Sept. 1979 to July 1980		Aug. 1980 to July 1981			

prepared. This stage required an average of 20 ± 4 min (mean ± s.d.) per diary. The other main functions were addressing the diaries (surprisingly time-consuming) and dealing with food items not found in *'The composition of foods'* (Paul & Southgate, 1978).

The three-day diaries (2269) were collected between September 1979 and July 1981 (100 weeks). The number of subjects involved is shown in Table 1. This averages 23 per week, higher if holidays are excluded. If some allowance is made for the planning period when a weighed pilot study was carried out, the coding was not finally complete until August 1981, and the data not finally ready for analysis until February 1982. If these periods are included, the collection rate becomes 14 three-day diaries per week on average. The high collection rate is attributable primarily to the use of the school organisation and to the inherent efficiency of the diary and interview method (Black, 1982).

Discussion

Transcription errors

Whenever data is manually transcribed errors will be made; ideally therefore all transcription should be verified. This was not possible in the present study, but an estimate of the number and type of errors was made. Ten diaries (30 days) were chosen at random from each of the five surveys and compared by another worker with their transcription sheets. These sheets had already been used for preparing punch cards; this stage was verified at considerable cost. Altogether 34 errors were found in the transcription of data from 150 days, or approximately one error in every 100 lines of data. The errors found were as follows: items missed out (usually of negligible nutritional value) 9 (26 per cent), incorrect time transcribed 7 (21 per cent), too few or too many intakes transcribed 6 (17 per cent), incorrect MW4 code 4 (12 per cent), incorrect weight 4 (12 per cent), administrative error 3 (9 per cent) and items transcribed in wrong order 1 (13 per cent). Only six of these errors were judged to require correction; approximately two in every 1000 lines of data. The most serious error (30 g boiled onion code 614 transcribed as 614 g crisps code 652 would have increased the subject's average intake by: protein 3 g, fat 15 g, carbohydrate 20 g, energy 929 kJ (222 kcal) and sugars 0.2 g. The effects of any remaining errors are difficult to evaluate, but those as serious as that shown above have been identified and corrected using a computer program written for this purpose. The error rate and types of error found (approximately one in every five days recorded) are unlikely to greatly affect the final data values, and would not therefore be expected to alter the conclusions drawn.

Validity

How valid are the data collected? A conclusive answer to this can never be known, since with all dietary surveys reliability and validity are difficult to assess (Young & Trulson, 1960). In an earlier investigation of the present study (Pearce *et al.*, 1981) 28 of the main study volunteers at three schools had one school meal assessed by the diary and interview method, and simultaneously by the weighed inventory method by another investigator; the two assessments were performed independently.

Table 2. *Average intake of school meals measured by either weighed inventory method (x) or by diary plus interview (y).* (Number of subjects 10 boys, 18 girls). There were no significant differences between the mean weighed and diary estimates ($P > 0.05$ by paired *t*-test). The low correlation coefficients suggest that the diary and interview method is a relatively inaccurate device for quantitatively estimating a schoolmeal intake of some individuals.

	n	*Weighed method* Mean	s.d.	*Dietary method* Mean	s.d.	*Correlation coefficients between x and y*	$\frac{y}{x} \times 100$
Energy kJ (kcal)	28	2330 (557)	43	2222 (531)	42	0.4*	95
Protein (g)	28	18	7	17	6	0.6**	94
Fat (g)	28	27	10	25	9	0.5**	94
Carbohydrate (g)	28	65	22	63	24	0.5**	97
Sugar (g)	28	22	13	21	11	0.6**	95

* $P < 0.05$; ** $P < 0.01$.

Good agreement was found between the mean nutrient intakes as estimated by the two methods (Table 2). It was also found that on several occasions that the diary and interview recorded more detailed qualitative information than weighing: the children often exchanged food which the second investigator did not discover, but which appeared in the diaries. However, correlation coefficients between the methods ranged from 0.6 to as low as 0.4. The conclusions from this small study were similar to those of many other workers (eg Bransby *et al.*, 1948; Maddern, Goodman & Gutherie, 1976). The diary and interview method may give a good estimate of the mean intake of a group of schoolchildren for both nutrients and foods. The low correlation coefficients suggest that the diary and interview method is a relatively imprecise device for quantitatively estimating a single meal intake of some individuals. This assumes that the weighed inventory method is itself accurate. However, it is possible that because of the consistent serving methods employed, the size of a school meal is easier to estimate than that of a meal taken at home. It must be emphasised that the correlation coefficients refer to one meal only, and when estimates of dietary intake are obtained for 15 days the reliability of the data will be very much higher (see section on reliability).

A further investigation related to the present study (Twist *et al.*, 1982) used the three-day diary and interview to estimate the three-day mean protein intake of 30 intelligent adults and correlate it to their 24-hour urine urea and ammonia excretion. A statistically significant, although relatively low correlation ($R = +0.43$, $P < 0.05$) was found. However, the three-day mean protein intakes from the diary (87.5 ± 3.5 g, mean ± 1 s.e.m.) were very similar to the estimate of protein intake made from the urea and ammonia excretion (86.6 ± 3,1 g, mean ± 1 s.e.m.) with suitable allowances for nitrogen excreted in other forms (urinary, faecal and dermal). From all of these results it was concluded that the diary and interview gave a valid estimation of the group's mean protein intake but again individual estimates may be imprecise.

Reliability

Table 3 shows, as percentages of the total variance in the observations of certain variables, the components attributable to different sources (Snedecor &

Table 3. *Percentage components of variance attributable to differences between children, surveys, and days for weights of sugars, protein, fat and carbohydrates consumed, and for total energy intake.* Also shown is the estimate of reliability based on these figures for five surveys of three-days each.

Variable	*Percentage of total variance attributable to:*			*Reliability (%)*
	Children	*Surveys*	*Days*	
Total sugars (g)	26	17	57	78
Protein (g)	25	15	60	78
Fat (g)	22	16	62	75
Carbohydrates (g)	29	18	53	80
Energy (kJ)	28	18	54	79

Cochran. 1971); from this it is possible to calculate the reliability of the estimate of the mean for an individual. It is interesting that the reliabilities for the various nutrients are so close. If the variances between children, survey, and days are σ^2_c, σ^2_s and σ^2_d respectively then the reliability (R) associated with a study of n_s surveys of n_d days each is

$$R = \sigma^2_c / \left\{ \sigma^2_c + \frac{\sigma^2_s}{n_s} + \frac{\sigma^2_d}{n_s n_d} \right\}$$

Table 4 shows the reliability of the estimate of a child's mean weight of sugar intake, based on the variances in Table 3, for studies of different strategies with regard to numbers of surveys and numbers of days per survey. In terms of the number of diaries collected, the most efficient strategy for high reliabilities tends to be surveys of two days each; for example, seven surveys of two days would have given a reliability of 80 per cent for weight of sugar intake, while five surveys of three days gave 78 per cent. Two surveys of seven days each would give a reliability coefficient of 0.68, highlighting the conclusion from Table 4 that replicating surveys yields greater precision than replicating days. However, in other ways it is more efficient to have fewer surveys of more days each, so five surveys of three days each is a good compromise.

Table 4. *Per cent reliability of the 'weight of total sugar intake per day', for varying number of surveys and days per surveys.*

		Number of surveys						
		1	2	3	4	5	10	15
Number of days per surveys	1	26	41	52	59	64	78	84
	2	37	54	63	70	74	85	90
	3	42	59	69	74	78	88	92
	4	46	63	72	77	81	89	93
	5	48	65	74	79	82	90	93
	7	51	68	76	81	84	91	94
	14	56	71	79	83	86	93	95

Acknowledgements—This study was supported by an M.R.C. project grant.

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Human Nutrition: Applied Nutrition (1985) **39A,** 167-177.

SOME INFLUENCES ON THE MEASUREMENT OF FOOD INTAKE DURING A DIETARY SURVEY OF ADOLESCENTS

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Data are presented from a recent longitudinal study of dietary habits of English children aged 11-13 y, in relation to various factors which may have influenced the estimations of intake. A knowledge of such relationships may aid the design and interpretation of this and other dietary surveys. The factors studied included survey fatigue, learning effect, season of the year, day of the week and the subjects' knowledge of the study's purpose. Generally the relationships found were of minor importance, but the variable influence of some of these factors is apparent from a study of the relevant literature. This is one reason why dietary survey methodology is resistant to simplification. It is concluded that the dietary survey method used was adequate for the purpose of the study.

Introduction

There have been many studies contributing to dietary survey methodology and several excellent reviews (Marr, 1971). However, it has been suggested that each new dietary survey, whatever the method employed, should include some attempt to assess the errors in the data produced (Young & Trulson, 1960). It cannot be assumed that to use a 'proven' method will inevitably produce useful results. The quality of the results will depend upon the method chosen, the subject participating, the design of the study, the type of data recorded and the investigator.

A large-scale longitudinal dietary survey of young adolescents has recently been completed which had the principal aim of relating estimates of each individual's mean dietary intake to their dental caries increment, both measured over the same 2-year period (Rugg-Gunn *et al.,* 1984). The highest correlation was found to be between the weight of total intake of sugars and caries increment. However, the size of the correlation was low and the possible reasons for this were discussed at length: intra-individual variation in dietary habits were found to be one contributory factor. It was suggested, therefore, that comparison between groups of subjects is a more informative method of relating diet to caries increment.

The design and conduct of the dietary study have been described in detail elsewhere (Hackett, Rugg-Gunn & Appleton, 1983) with particular emphasis being placed on the constraints which determined the study design, and evaluation of some of the sources of error associated with the method. The reliability of the dietary data was of the order of 80 per cent for macronutrients. Furthermore, the dietary findings agreed with those of previously published surveys which used more elaborate survey techniques (Hackett *et al.,* 1984*a*). Despite these findings, no relationship between

nutrient intake and the growth of these adolescents could be found (Hackett *et al.*, 1984*b*). Methodology was suggested as the most likely explanation; in particular, a very high reliability of the measurements would be required as it was calculated that only approximately 3 per cent of energy intake was used for growth at this age. The dietary sources and intake of sugars (Hackett *et al.*, 1984*c*), and calcium (Hackett *et al.*, 1984*d*), have also been reported in detail.

This paper uses the data collected during the survey to assess other considerations which affect the design and interpretation of dietary surveys.

Subjects and methods

Main study

The volunteers for this survey were recruited from seven of the eight middle schools in the Morpeth, Ashington and Newbiggin area of Northumberland. The eighth school was unavailable due to an extensive rebuilding programme. Between September 1979 and July 1981, 193 boys and 212 girls, initially aged between 11 and 12 years, each recorded their dietary intake in specially designed diaries on five occasions each of 3 consecutive days' duration. All the children were presumably aware of the purpose of the study since their parents were fully informed in a letter seeking consent to their child's participation. Each child was dentally examined in October 1979, 1980 and 1981. A record of all foods and drinks consumed during each 3-day period was requested and each child was interviewed on the 4th day by one nutritionist to verify and enlarge upon the information provided. By questioning, and with the aid of food models, a reasonably reliable quantitative record of food intake was obtained (Hackett *et al.*, 1983). The diaries were distributed and the interviews conducted at school. Each child was told on which days to complete the diary, and the days were chosen with the aim of ensuring that all days of the week were included during the 2-year study period. Because interviews were not conducted at home, nearly all records referred to term time only, although information was obtained for all months of the year.

All the information collected was stored in a computer and the weights of food consumed were analysed using computerised food tables (Paul & Southgate, 1978). These food tables were modified and supplemented as required. The occupation of the father of each child was also recorded and encoded as social class (Registrar General, 1970).

Subsidiary study

It was decided to conduct a subsidiary study to investigate whether knowledge of the dental aims of the study influenced the subjects' recording of their dietary intake. For example, the well-publicised relationship between sweet-eating and dental decay might lead to under-reporting of the intake of sweets. Despite the rebuilding programme, it was possible to conduct this study of one 3-day diary at the remaining (eighth) school in the area, during July 1980. The children were invited to volunteer for a dietary survey only, which they were told was to be conducted by staff from the medical faculty of

the University. They were not dentally examined. Because food intake may be influenced by a variety of factors, each of the children in this study was matched, twice, to children who had completed the main study. They were matched (in order of priority) for: age, sex, race, social class, school stream and locality. Season of the year was also taken into account by using the second of the diaries recorded by the main survey children (kept during Spring or Summer 1980), which was therefore compared to the first (and only) diary kept by the subsidiary study children. It was assumed therefore that any 'first diary effect' — see Results — was likely to be smaller than the effect of season of the year.

Results

The results presented are limited mainly to the intake of energy, protein and sugars.

Day of survey

Each survey lasted for 3 consecutive days: the mean intakes on each of these days for the 193 boys and 212 girls are shown in Table 1. There were no significant differences in any of the intakes between the 3 days. The Pearson correlation coefficients between the energy intakes on each pair of these days (for the first survey only) were as follows: day 1 and 2, $r = +0.44$; day 1 and 3, $r = +0.34$; and day 2 and 3, $r = +0.35$ ($P < 0.01$ in each case). A similar value (0.34) was found for the within-survey reliability using methods previously described (Hackett *et al.*, 1983) of estimating one day's energy intake. This confirms the large variance in any one individual's intake and the value of multiple surveys (Hackett *et al.*, 1983).

Table 1. *Intake of energy, protein and sugars by day of survey.* Data for all five surveys included. Total number of days = 6075 (405 subjects × 15 days). The s.e.ms were very similar and were as follows: energy — boys 0.8, girls 0.7 MJ; protein — boys and girls 0.6 g; sugars — boys 1.6, girls 1.5 g.

	Day of survey		
	1st	*2nd*	*3rd*
Males (n = 193)			
Energy (MJ)	9.6	9.4	9.5
Protein (g)	64	64	64
Sugars (g)	124	124	124
Females (n = 212)			
Energy (MJ)	8.5	8.5	8.4
Protein (g)	55	56	56
Sugars (g)	113	115	112

Day of week

The distribution of the days surveyed is shown in Table 2. Relatively few Thursdays and Fridays were studied because no interview was conducted on Saturday or Sunday. For the same reason, day 1 of the survey was seldom

Table 2. *Distribution of all days surveyed and Day 1 only, by day of week*

	All days		*Day 1*
	Number	*%*	*%*
Monday	1122	18.5	20.2
Tuesday	1121	18.4	19.7
Wednesday	812	13.4	0.1
Thursday	464	7.6	3.2
Friday	511	8.4	22.0
Saturday	896	14.7	19.1
Sunday	1149	18.9	15.7
	6075	100	100

Wednesday or Thursday, day 2 seldom Thursday or Friday and day 3 seldom Friday or Saturday.

The mean intakes for each day of the week, pooled for 5 surveys, are shown in Table 3. An analysis of variance was carried out and large differences ($P < 0.01$) between the sexes were found: these have been reported in more detail elsewhere (Hackett *et al.*, 1984*a*). Similarly, an increase in intake over the 5 surveys was found, together with a 'sex and survey' interaction ($P < 0.05$). That is the boys increased their already higher intakes more than the girls. No interaction between sex and weekdays was found; the boys and girls showing a similar weekday pattern with the highest intake on Saturdays and/or Sundays. No interaction between surveys and days of the week was found except for a marginally significant interaction for protein intake; the pattern otherwise remaining similar over the five surveys. Furthermore, no interaction between sex, survey and weekday was found.

Table 3. *Intake by day of week.* Data for all five surveys included. Values are means (± s.e.m.)

Males	*Mon.*	*Tues.*	*Wed.*	*Thurs.*	*Fri.*	*Sat.*	*Sun.*
Energy (MJ)	9.2 (0.1)	9.5 (0.1)	9.5 (0.1)	9.6 (0.2)	9.5 (0.1)	9.7 (0.1)	9.4 (0.1)
Protein (g)	61 (0.8)	64 (0.9)	65 (1.3)	65 (1.3)	63 (1.1)	64 (1.0)	67 (0.9)
Sugars (g)	116 (2.1)	121 (2.1)	122 (2.5)	126 (2.3)	121 (3.1)	127 (2.7)	133 (2.3)
Females							
Energy (MJ)	8.3 (0.1)	8.3 (0.1)	8.3 (0.1)	8.4 (0.2)	8.5 (0.1)	8.8 (0.1)	8.6 (0.1)
Protein (g)	54 (0.7)	54 (0.7)	55 (0.9)	55 (1.3)	54 (1.1)	56 (0.9)	60 (0.8)
Sugars (g)	108 (1.8)	109 (1.9)	108 (2.3)	109 (3.0)	111 (3.1)	118 (2.6)	125 (2.2)

Season of the year

The intakes of energy, protein and sugars are shown according to season of the year in the Figure. Spring was taken as being March, April and May; summer as June, July and August; autumn as September, October and November and winter as December, January and February. All the seasons

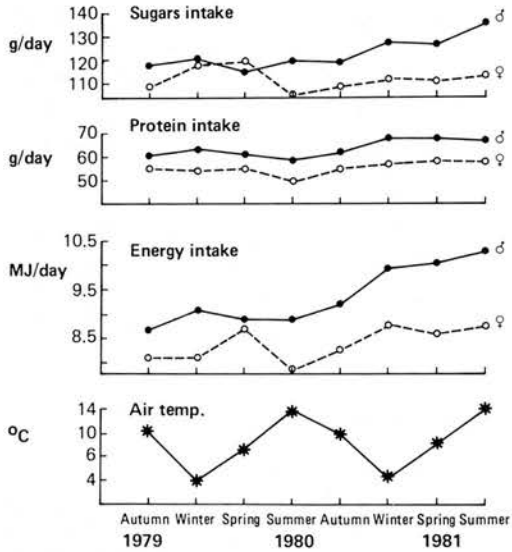

Figure. *Average daily intakes of energy, protein and sugars, and average air temperature according to season of the year.* ●, *boys;* ○, *girls.*

are of three months except summer 1981 for which data are available only for June and July. There were also few data available for August 1980. The mean air temperatures recorded in Newcastle upon Tyne for these months are also given. There is clearly no strong relationship between intake and season.

The five surveys

Intakes of energy, protein and sugars recorded in each of the 5 surveys, previously reported by Hackett *et al.*, 1984*a*, are summarized in Table 4. The boys increased their energy intake by approximately 13 per cent over the 2 years, whilst girls increased their intake by about 7 per cent (by regression analysis). The percentage contribution of the components to the energy intake (protein, fat and carbohydrate) remained approximately constant.

Table 4. *Average daily intake of energy, protein and sugars according to survey and sex.* The coefficients of variation of the means were virtually the same for either sex at each survey, and were approximately: energy 22 per cent, protein 24 per cent, and sugars 33 per cent.

	Survey				
	I	*II*	*III*	*IV*	*V*
Energy (MJ)					
Boys	8.9	8.9	9.3	10.0	10.3
Girls	8.1	8.4	8.3	8.7	8.8
Protein (g)					
Boys	62	60	63	68	68
Girls	54	53	55	56	59
Sugars (g)					
Boys	121	118	122	126	133
Girls	114	116	109	111	116

The Pearson's correlation coefficients between a child's energy intake at each of the five surveys and the same child's intake at each of the other four surveys are shown in Table 5. There was a trend towards a decrease in correlation as the interval between surveys lengthened: for example, the correlation between surveys I and II was ±0.41 while between I and V it was +0.33.

Table 5. *Pearson's corelation coefficients (r) between the energy intake at each survey and that at each other survey.* All were positive with $P < 0.01$.

	Survey			
	II	III	IV	V
I	0.411	0.378	0.419	0.329
II		0.511	0.453	0.454
III			0.545	0.530
IV				0.540

Table 6 shows the square root of the 'between-surveys' variance for several nutrients, calculated both with and without the first survey. The variance for energy decreased slightly when only four surveys were used, but the differences were small and not all components were equally affected.

Table 6. *Square root of the 'between surveys' component variance calculated with and without the inclusion of the first survey.*

	All five surveys	*Last four surveys*
Energy	1108	1055
Protein	8	11
Fat	14	12
Carbohydrate	35	35
Sugars	21	20

Subsidiary study

A summary of the results of the one survey carried out at the eighth ('control') school compared to the intakes of the two matched groups of children from the main study ('Group 1' and 'Group 2') is shown in Table 7. Twenty-five boys and 27 girls completed one diary at the 'control' school and were twice matched to the same number of boys and girls from the main survey. Even with over 400 children completing the main study, only 2 complete sets of children were found which were comparable to the children of the subsidiary study. There were no statistically significant differences ($P > 0.05$, *t*-test) between the intakes of Gp 1 and Gp 2 (indicating the success of the matching procedure) or between either Gp 1 or Gp 2 and the control children's intake ($P > 0.05$, *t*-test). The power of this study to detect differences was low; differences of 0.5 MJ, 30 g sugars, 30 g confectionery and 0.9 intakes of confectionery per day would be required to be 95 per cent certain that a difference between the mean intake was significant at the 5 per cent level in samples of this size.

Table 7. *Comparison of intakes between children dentally examined and aware of the reasons for the study (Gp 1 and Gp 2) and children unaware of the reasons for the study (control)* Figures given are mean values. For all comparisons between group 1 and Control and between group 2 and Control $P > 0.05$.

	Group 1 ($n = 52$)	Control ($n = 52$)	Group 2 ($n = 52$)
Energy (MJ)	8.9	8.2	8.6
Protein (g)	55	54	58
Fat (g)	98	89	94
Carbohydrate (g)	273	252	262
Total sugars (g)	118	116	116
Confectionery (g)*	59 (1.69)	54 (1.89)	51 (1.61)
Sweet biscuits and puddings (g)*	125 (1.39)	102 (1.58)	127 (1.58)
Sweet drinks (g)*	388 (2.06)	347 (2.37)	322 (2.04)

*With frequency of intake in parentheses.

A large number of statistical tests have been applied to these data and caution is therefore required when discussing the results.

Discussion

The design of the dietary survey reported here was necessarily complex and not always optimal. The data have therefore been examined in order to try to locate sources of variability. One possible large source of variability was not present in this study as only one nutritionist conducted all the dietary survey work.

Young & Trulson (1960) commented that the length of dietary surveys should not be prolonged lest the interest and co-operation of the subjects be lost. If these problems had occurred in the present study the energy intake might have been expected to have decreased over the 3 days, and the coefficient of variation of the energy intake on succeeding days to have increased. Neither of these effects was found. This finding is similar to those of Durnin *et al.* (1974) and Heady (1961) and suggests that the enthusiasm of the children was maintained.

A relationship between day of the week and food intake has not been found consistently in other surveys: Beaton *et al.* (1979) observed that working women, but not men, ate 'more food' on Sundays than on weekdays. Heady (1961) reported little evidence of an effect on the nutrient intakes of bank clerks although some foods were more popular on certain days of the week. Durnin *et al.* (1974) found no difference in the protein or energy intake of adolescents between weekdays and Saturdays or Sundays, but Eppright *et al.* (1952) concluded that the weekend eating habits of the children they studied were likely to differ significantly from those on school-days. Finally, the results of Chalmers *et al.* (1952) showed no effect of day of the week in their varied groups of children, except in college children who had lower intakes at weekends. In the present study the nutrient intake at the weekend was different compared to weekdays, but Saturdays and Sundays

did not differ in quite the same way. It is concluded that the effect of days of the week on intake is unpredictable and therefore all days should be sampled in dietary studies.

The season might be expected to affect food intake by either altering food availability, or changing behaviour or physiology. However, in several studies there is no evidence of a major influence by season on nutrient intake. Young, Lightbody-Smudski & Steele (1951) found little effect on the nutrients they studied even in rural areas. According to Eppright *et al.* (1952), there were no consistent seasonal differences although protein and iron intake tended to be slightly greater in the autumn and ascorbic acid intake higher in the spring. Heady (1961) concluded that there were no important seasonal differences in energy, protein or fat intake, but that there were differences in the foods eaten. Balogh, Kahn & Medalie (1971) reported an increase in the coefficient of variation as they increased the number of monthly dietary surveys of government employees, attributing this, in part, to seasonal variation. Examination of their data reveals that this increase in variation did not apply equally to all the nutrients studied. Beaton *et al.* (1979) concluded that their survey of the intakes of sales and office staff implied the absence of any effect of season. Marr (1971) reviewed several surveys and found that nutrient intake has been reported to be higher in either winter or summer months. Interpretation of the results from the present survey is complicated by the relatively few subjects studied during the summer months and the tendency for intake to rise over the 2-year period. Seasonal effect on the intake of some nutrients (energy, protein and sugars) seems to be small and, though variable, is unlikely to complicate many dietary surveys. However, the intake of some foods does show a strong seasonal variation. Season should be borne in mind when comparing results between surveys within a large cross-sectional study or when calculating an individual's mean intake from a longitudinal survey.

In the present study the energy intake of the boys increased by about 13 per cent over the 2 years and that of the girls by only 7 per cent. Calculations from Widdowson's (1947) cross-sectional study show that the 13-year-old boys and girls consumed about 9 per cent more energy than the 11-year-olds. Similarly, the longitudinal study of Burke *et al.* (1959) showed that boys aged 13.5 years had energy intakes 19 per cent greater than when they were aged 11.5 years, and that for girls there was a 5 per cent increase. The longer a longitudinal study lasts, especially with children or adolescents, the less appropriate is the calculation of an individual's mean intake.

To improve the reliability of a dietary survey method it is better to take more surveys than more consecutive days (Chappell, 1955; Hackett *et al.*, 1983), but increasing the number of surveys disproportionately increases the burden on the subjects. Topp, Cook & Elliot (1972) concluded that mothers were probably trying harder to provide nutritious meals during the first week of their 4-week study of children. A learning effect may also be apparent: Meredith *et al.* (1951) suggested that the children whom they interviewed later were better prepared (presumably by discussion with their peers) for the recall sessions than those seen earlier. In the present study

there was a noticeable improvement in the keeping of the diaries after the first survey and the interviews were shorter and smoother although this was not reflected in a change in energy intake. Beaton *et al.* (1979), however, found no suggestion of either a training effect or a reaction against repeated dietary interviews. They interviewed each subject six times over an interval of 2.5 to 14 weeks. The possibility that the first survey in the present study was in some way biased was considered; the children might have kept their diaries particularly diligently the first time or perhaps their interest waned over the 2 years. Conversely their records might have improved after the first diary once they were aware of exactly what to do (and perhaps confident of a lack of retribution). The energy intakes at the first survey did seem proportionately higher than for the other surveys, especially for the boys. Also the correlation coefficients were a little lower between the first survey and the others than for any other combination of surveys. Furthermore, the component variances did decrease slightly if only the latter four surveys were used, but not by much and not for all nutrients. The relationships between the dietary factors measured and the dental caries increment were not affected by using the last four rather than all five surveys. It is therefore concluded that any 'first survey effect' was not important for the factors investigated.

Dental research workers have expressed concern that dietary data recorded in surveys of diet and dental disease may be biased because the subjects are likely to have a knowledge of the dietary causes of dental disease (Martinsson, 1972). Burke (1947) discussed the problem of creating an artificial situation, with the subjects not eating as they normally do. Leitch & Aitken (1950) also acknowledge the possibility of such a problem, while Young & Trulson (1960) reported the possibility that a subject may either misreport or actually change his intake for the duration of the study. Despite these suggestions, it appears that no one has previously attempted to quantify this bias. In the present study there were no significant differences between those children aware of the dental origin of the study and those unaware. However, the groups differed from the control children, who, although recruited in the same way, were approached much later in the school year (June 1980). Also the volunteer rate was lower (37 per cent compared with 59 per cent), but the social class distributions of the volunteers were similar. Finally, the control survey was carried out during the hottest 2 weeks of that school year. These difficulties were unsurmountable under the strict timetable necessary to complete the main survey. Furthermore, the power of this test was also found to be low. It is therefore only tentatively concluded that there was no large additional bias created by the dental aspect of the main study.

Young & Trulson (1960) assert that if possible at least modest attempts should be made to test dietary survey methods whenever they are used for epidemiological studies. Woolf (1954) discussed in detail the shortcomings of dietary surveys and concluded that they are a 'rather rough instrument'. He also pointed out that minimising one particular error is of little value if other equally large errors exist.

We have reviewed here some suspected sources of error and bias in a large scale longitudinal study of adolescents. Most errors examined were found to be minor and especially so when compared to the problems involved in studying a large representative sample, using food tables. Wiehl & Reed (1960) pointed out that no one dietary survey method is best for all situations and purposes: the 3-day diary and interview method was found to be suitable for studying the macronutrient and sugars intake of young adolescents.

Acknowledgements—This work was supported by an MRC project grant.

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A 2-year longitudinal nutritional survey of 405 Northumberland children initially aged 11·5 years

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(*Received 7 April 1983 – Accepted 12 August 1983*)

1. Children (405), initially of average age 11·5 years, recorded all food and drinks consumed for three consecutive days (with an interview on the fourth day) on five separate occasions over a 2-year period. Food tables (Paul & Southgate, 1978) enabled nutrient intakes to be calculated. The data collected were found to be of high reliability (Hackett *et al.* 1983).

2. The mean energy intakes showed broad agreement with other recent British dietary surveys but were well below those recorded in the prewar study by Widdowson (1947) and the FAO/WHO (1973) recommended levels. They were slightly below the current Department of Health and Social Security (DHSS; 1979) recommended intakes.

3. Over the 2-year period, the energy intake of the boys increased by 13% compared with an increase of only 7% in the girls.

4. The iron and vitamin A intakes of all groups of children were low compared with current recommendations (DHSS, 1979). This seems to be a result of falling energy intake. Mean calcium intakes were also only marginally in excess of the recommended intake, and those of most of the girls would fall below the recommendation if the proposal to end the fortification of flour (DHSS, 1981) is implemented.

The purpose of this study of a large group of young adolescents was to relate an estimate of each individual's mean dietary habits to his or her observed dental caries increment. However, the nutritional information collected is of wider interest and is presented in the present paper as it gives a recent estimate of intake of a group of northern English schoolchildren from a variety of backgrounds. It is also unique amongst British surveys, being longitudinal in design.

SUBJECTS AND METHODS

In July 1979, seven middle schools in the Morpeth, Ashington and Newbiggin area of South Northumberland, chosen to give a representative range of social classes, were invited to participate in the study. All third-year children, initially aged 11–12 years old, were asked by their headmasters to volunteer for the survey which was aimed at relating their dietary habits to their dental caries increment. The study was also described in a letter to their parents, who were asked to consent to their child taking part. Those who did not volunteer were not approached further. Ethical approval for all aspects of the study was obtained from the Northumberland Area Health Authority.

Each child was dentally examined (including bite-wing radiographs) in October 1979, 1980 and 1981, in a purpose-built mobile examining van. Their heights and weights (without shoes and jackets) were also measured at these times by the same person. A discussion of the children's height, weight and growth in relation to their diet will be published separately.

On five occasions between September 1979 and July 1981, each child recorded, in a specially-designed diary, all foods and drinks taken for three consecutive days. The children

* For reprints.

Table 1. *Summary of subjects taking part*

	No.	%	
Total no. of second-year children	784	100	—
Volunteers	466	59·4	100
Moved or absent for one or more surveys	54	6·9	11·6
Withdrew	2	0·3	0·4
Rejected	5	0·6	1·1
Completed all aspects of survey	405	51·7	86·9

Table 2. *Subjects grouped into social class by father's occupation (Registrar General, 1970)*

Social class	No.		%	
High				
I	20	85	5	21
II	65		16	
Middle				
III	172		43	
Low				
IV	96	118	24	29
V	22		5	
VI, VII and VIII	30		7	
Total	405		100	

also recorded their times of consumption, estimations in household measures of the quantity eaten, whether each item was eaten alone or in combination with any other item, and their bedtimes. They were each then interviewed on the fourth day by one nutritionist, to verify and enlarge on the information provided. By questioning and with the aid of food models, a quantitative record of food intake is thought to have been obtained (Hackett *et al.* 1982) and the method has been described in more detail (Hackett *et al.* 1983).

All the information collected was stored in a computer and the weights of food consumed were analysed using computerized food tables (Paul & Southgate, 1978). The food tables were modified and supplemented as required.

The occupation of the father of each child was also recorded and coded as social class (Registrar General, 1970). Social classes I and II were then labelled 'high', III as 'middle' and IV and V as 'low'. Groups VI (unclassifiable), VII (retired or unemployed) and VIII (unknown) were excluded from any analysis by social class due to the small number of subjects involved.

As the standard deviations showed a tendency to vary with the means, the coefficient of variation for each variable is given as the measure of variation. To allow for the possibility of skewness in the results, when comparing the nutrient intakes to the recommended daily allowances (RDA) the number of subjects falling below the RDA has been calculated.

Differences in mean intake between social classes were tested for statistical significance by the analysis of variance and differences in proportions of children below the RDA by χ^2 analysis of 3×2 contingency tables.

Table 3. *Average daily macronutrient and sugars intake by survey and sex*

Survey...	1		2		3		4		5	
Date	Sept 1979–Feb 1980		Feb 1980–July 1980		Sept 1980–Dec 1980		Dec 1980–Apr 1981		Apr 1981–July 1981	
Sex	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Energy (MJ)	8·9	8·1	8·9	8·4	9·3	8·3	10·0	8·7	10·3	8·8
Protein (g)	62·3	54·4	60·2	53·1	63·1	54·9	67·7	56·5	68·3	58·8
Fat (g)	92·6	86·6	97·1	93·3	99·5	90·8	109·6	97·1	108·5	94·8
Carbohydrate (g)	273·8	248·8	269·1	254·3	284·7	251·7	300·0	257·3	316·9	268·3
Sugars (g)	121·0	114·0	117·7	115·9	121·8	109·2	126·4	111·4	132·6	115·9

Coefficients of variation were virtually the same for either sex at each survey and were approximately: energy 22%, protein 24%, fat 25%, carbohydrate 23%, sugars 33%.

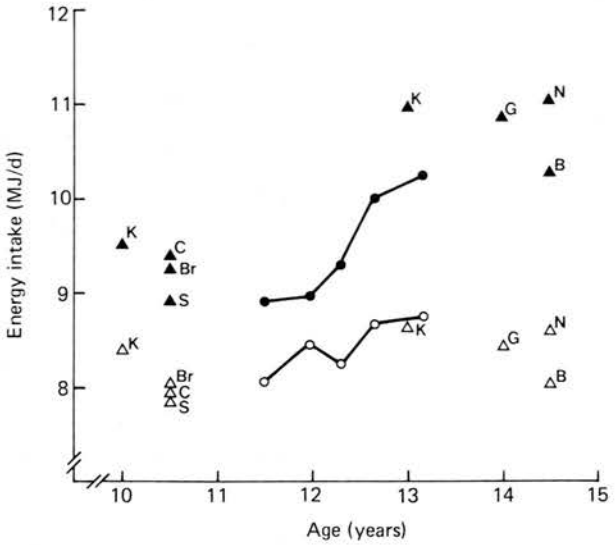

Fig. 1. Average energy intake on five occasions over a 2-year period of 193 boys (●) and 212 girls (○) in Northumberland compared with some other published British surveys of boys (▲) and girls (△) in Kent (▲K, △K) (Cook *et al.* 1973), Glasgow (▲G, △G) (Durnin *et al.* 1974), Newcastle (▲N, △N) and Birmingham (▲B, △B) (Darke *et al.* 1980) and Bristol (▲Br, △Br), Croydon (▲C, △C) and Sheffield (▲S, △S) (Darke & Disselduff, 1981).

RESULTS

Of those asked, 466 children (59·4%) volunteered. This rather low acceptance rate may be related to the subsequent extremely low drop-out rate (0·4%, Table 1). All aspects of the study were completed by 212 girls and 193 boys. The social class distribution (Table 2) showed that despite the low volunteer rate, the various social classes were all well represented.

The mean dietary intakes of the boys and girls at each of the five surveys are shown in Table 3. The boys ate consistently more than the girls and the difference became more pronounced over the 2-year study period: the boys increased their average energy intake by about 13% while that of the girls increased by about 7% (estimates by regression analysis). Fig. 1 shows the energy intake for the boys and girls for each of the five surveys plotted alongside results from other recent British surveys of young adolescents in Kent (Cook *et al.* 1973), Glasgow (Durnin *et al.* 1974), Newcastle and Birmingham (Darke *et al.* 1980) and Bristol, Croydon and Sheffield (Darke & Disselduff, 1981). All these authors used the 7 d weighed inventory method. The average energy intake of the children in the present study corresponded with the intakes reported by these other authors.

The percentage contribution of the macronutrients (protein, fat and carbohydrate) and sugars to the average energy intake is shown in Table 4. The compositions of the diets were very similar both between the sexes and between the surveys. The large contribution of sugars can be seen; lactose contributed 15·2 g/d (13%) of the total sugars intake. Further breakdown of the sugars intake has not been attempted.

Table 5 shows the macronutrient and sugars intakes for each sex and social class: there were few statistically-significant differences between social classes. More middle-social-class girls had energy intakes below the Department of Health and Social Security (DHSS, 1979) RDA than the other girls. The high-social-class boys consumed more lactose than the low- or middle-social-class boys.

Table 4. *Percentage contribution of macronutrients and sugars to energy intake (from the mean of all five surveys)*

	Percentage of energy intake	
Sex...	♂	♀
Protein	12	11
Fat	40	40
Carbohydrate		
Total	49	48
Sugars	21	21

Table 5. *Average daily macronutrient and sugars intake by sex and social class compared with recommended intakes (DHSS, 1979)*

(No. of children in parentheses)

	Energy		Protein					
Social class	MJ	% below RDA	g	% below RDA	Fat (g)	Carbo-hydrate (g)	Sugars (g)	Lactose (g)
♂								
High (46)	9·4	91	65	48	102	283	127	20
Middle (86)	9·4	85	63	59	100	286	120	16
Low (52)	9·5	81	64	56	102	290	124	17
Significance	NS	NS	NS	NS	NS	NS	NS	*
RDA	11·0	—	66	—	—	—	—	—
♀								
High (39)	8·6	54	58	38	95	256	119	14
Middle (86)	8·3	77	54	44	89	252	113	14
Low (66)	8·6	59	55	44	95	261	111	12
Significance	NS	*	NS	NS	NS	NS	NS	NS
RDA	9·0	—	53	—	—	—	—	—

RDA, recommended daily allowance; DHSS, Department of Health and Social Security.

The coefficients of variation were similar between sexes and social classes with values of approximately: energy 15%, protein 17%, fat 18%, carbohydrate 17%, sugars 24%, lactose 42%.

NS, not significant; * $P < 0{\cdot}05$.

The average intakes of some other nutrients compared with the RDA are shown in Table 6. There were no differences in vitamin A intake between the social classes. The higher-social-class girls had a higher Ca intake than the lower-social-class girls and a higher Fe intake than the middle-social-class girls. The lower-social-class girls also had higher Fe intakes than the middle-social-class girls. Few children had intakes of vitamin C below the RDA, but more girls than boys had Ca intakes below the RDA. In contrast most boys and girls had Fe and vitamin A intakes below the RDA.

Table 7 shows the intake of nutrients per unit energy, giving an index of the quality of the diet (or nutrient density). The Ca intakes per MJ of the higher-social-class boys were higher than those of either the middle- or lower-social-class boys, and the Ca intakes per MJ of the higher- and middle-social-class girls were each higher than those of the lower-social-class girls. The intake of Fe per MJ was higher for the higher-social-class girls than either the middle- or lower-social-class girls.

These few significant results have to be treated with caution because of the large number

Table 6. *Average daily intakes of some nutrients by sex and social class compared with recommended intakes (DHSS, 1979)*

(No. of children in parentheses)

Social class	Fibre g	Calcium mg	Calcium % below RDA	Iron mg	Iron % below RDA	Vitamin A (retinol equivalents) μg	Vitamin A % below RDA	Vitamin C mg	Vitamin C % below RDA
♂									
High (46)	14·6	953	13	10·3	80	721	61	43·3	15
Middle (86)	14·6	864	26	10·8	70	730	62	40·1	14
Low (52)	14·9	871	17	10·6	75	644	71	37·9	15
Significance	NS	NS	NS	NS	NS	NS	NS	NS	NS
RDA	—	700	—	12·0	—	725†	—	25·0	—
♂									
High (39)	14·2	836	28	10·1	85	654	61	41·0	13
Middle (86)	13·0	780	34	9·0	93	609	74	40·9	14
Low (66)	13·6	742	42	9·6	91	569	76	37·9	14
Significance	NS	*	NS	**	NS	NS	NS	NS	NS
RDA	—	700	—	12·0	—	725†	—	25·0	—

RDA, recommended daily allowance; DHSS, Department of Health and Social Security.

The coefficients of variation were similar between the sexes and social classes and were approximately: fibre 24%, Ca 24%, Fe 19%, vitamin A 60%, vitamin C 40%.

† The method used by the DHSS to calculate these values may give results about 7% higher than if these values had been calculated using the method of Paul & Southgate (1978). This latter method was used to calculate intakes in the present study (the RDA is estimated at approximately 675 μg by this method).

NS, not significant; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

Table 7. *Average daily intake of nutrients (/MJ energy) by sex and social class*

(No. of children in parentheses)

Social class	Protein (g)	Fibre (g)	Calcium (g)	Iron (mg)	Vitamin A (retinol equivalents) (μg)	Vitamin C (mg)
♂						
High (46)	7·0	1·5	100	1·09	75	4·6
Middle (86)	6·9	1·6	92	1·15	77	4·3
Low (52)	6·8	1·6	92	1·12	68	4·0
Significance	NS	NS	*	NS	NS	NS
♀						
High (39)	6·8	1·7	98	1·18	76	4·8
Middle (86)	6·6	1·6	94	1·09	74	5·0
Low (66)	6·5	1·6	86	1·12	65	4·5
Significance	*	NS	**	**	NS	NS

The coefficients of variation were similar between the sexes and social classes and were approximately: protein 10%, fibre 18%, Ca 19%, Fe 11%, vitamin A 56%, vitamin C 39%.

NS, not significant; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

of tests carried out and only those results considered to have practical nutritional significance will be discussed further.

DISCUSSION

The 3 d diary and interview method of measuring dietary intake has proved to be an acceptable and efficient means of collecting a very large amount of data from young adolescents of both sexes and all social classes. Using this method, dietary data of high reliability (e.g. reliability coefficient for energy 0·8) has been obtained (Goldstein, 1979; Appleton *et al.* 1983; Hackett *et al.* 1983). The nutrient intakes recorded were in broad agreement with other recent British dietary surveys for children of a similar age-range (Fig. 1) and confirm the long-term downward trend in energy intake (Whitehead *et al.* 1982). The agreement between the present study and the other recent British surveys of children of this age-group further suggests the value of this relatively cheap and simple survey method.

The divergence in the boys' and girls' energy intakes over the 2-year period is similar to the patterns reported by Burke *et al.* (1959) from a longitudinal study, and to that found in the extensive cross-sectional study of Widdowson (1947). However, the actual values recorded are well below those found in these earlier studies. Like these studies, the energy intakes of the boys and girls showed different patterns; the intake of the girls increased considerably less steeply than that of the boys. These differences may be related to the stage of puberty reached, the girls being nearer 'maturity'.

The children's energy intakes were approximately 20% below the FAO/WHO (1973) recommendations, suggesting that these recommendations are not suitable for British children. The average energy intake of the boys was about 14% and that of the girls 5% below the RDA (most of the boys had energy intakes below the RDA but the reasons are not clear). The children might have been under-reporting their intake slightly and the boys' intake being proportionately lower than that of the girls' may support this. However, the DHSS (1979) RDA may be currently a little too high since there has been a downward trend in energy intake for several decades (Whitehead *et al.* 1982). The children's average protein intakes were similar to the RDA.

Over 20% of the children's energy intake was derived from sugars. The increased risk of the development of dental caries from a large sugar intake is generally recognized (DHSS, 1978). Only 10% of the total energy intake from refined or processed sugar has been recommended (United States Senate, 1977). The consumption of less sugars, particularly less table sugar, which apart from carbohydrate is nutrient-free, would be likely to improve the quality of the diet consumed. The dental implications of the sugars intake of these children will be discussed elsewhere. Apart from sugars, the proportion of energy consumed as protein, fat and carbohydrate agreed with current recommendations (DHSS, 1979).

The average Fe intakes of most individuals and all groups of boys and girls were below the RDA. To achieve an intake of 12 mg Fe/d the girls would need to have consumed a diet supplying 1·5 mg Fe/MJ, which is impractical (DHSS, 1979). Anaemia is usually prevented by an intake of 1·3 mg Fe/MJ (DHSS, 1979), a value 15% above the actual average consumption of these groups and above the intakes of most of the groups of adolescents in the British surveys previously listed. Alternatively, the girls would need to have consumed nearly one-third more energy from a diet of similar quality. Energy intake, therefore, appears to be limiting their Fe intake. The Fe intake of the girls was similar to that found in 1970 by Darke *et al.* (1980) in eighty-five 14·5-year-old Newcastle girls (10·9 mg/d) but whose diets were richer in sources of Fe (1·3 mg/MJ). However, a low estimated Fe intake may not necessarily indicate cause for concern as the use of food tables can give rise to very poor estimates of Fe intake (Marr, 1971). Furthermore, a clinical and haematological study is essential before a diagnosis of Fe-deficiency anaemia can be made.

Fe-deficient erythropoiesis has been suggested as a problem during adolescence in 15% of boys living in poverty and 20% of all girls (Marshall, 1976).

The mean Ca intakes were above the RDA and similar to the Ca intakes recorded in the other surveys of adolescents previously discussed. The Ca intakes in the 1971 survey of Durnin *et al.* (1974) are below those recorded by their earlier survey in 1964 (in different children). Possibly Ca intake is falling and, as with Fe, it may be limited by the energy intake. If this is so the proposed removal of the requirement to add Ca to white flour may be to the detriment of those children with the lowest intakes (lower-social-class girls in particular). If the flour consumed in the present study had not been fortified, Ca intakes would have been about 16% lower, the mean intakes of both the middle- and lower-social-class girls falling below the RDA and the mean intake of the high-class girls equalling it. This will be discussed fully elsewhere.

Intakes of vitamin A of most of the girls and boys were below the RDA. This again may be related as much to falling energy intake as to consumption of a poor-quality diet. Calculations from the studies of adolescents previously listed suggest that little change has occurred in the quality of the diet of adolescents over recent years. Calculations from the information collected by Widdowson (1947) between 1935 and 1939 for 12- and 13-year-olds gave the following intakes for boys and girls respectively: protein (g/MJ) 6·8, 6·9; Ca (mg/MJ) 65, 65; Fe (mg/MJ) 1·2, 1·2; vitamin C (mg/MJ) 5·1, 4·4. This suggests that although there has been a marked improvement in the quality of the diet with regard to Ca (in part due to the fortification of flour introduced in 1942) there have been few changes in the quality of the diet in general. This is in agreement with the thesis of Truswell & Darnton-Hill (1981) that the diet of the modern adolescent is not as bad as is commonly thought. However, the progressive fall in energy intake may still lead to a fall in nutrient intakes.

The fibre intake of all groups of children was low compared with the value of 20 g/d found for a typical UK diet (Bingham & Cummings, 1980) and the suggested recommendation of 40 g/d (Stephen, 1981). It has been suggested that bread, fruit and vegetable intakes be increased and that an increased intake of cereal fibre may be beneficial (DHSS, 1981). The maximum value recorded for an individual in the present study (28 g) suggests that 40 g may be an unrealistic recommendation for children of this age at the present time.

As has been found in other studies of adolescents (Cook *et al.* 1973; Durnin *et al.* 1974) there were few, relatively minor differences in macronutrient intake between the social classes. This is in contrast to a study of preschool children which found that the lower-social-class children consumed more energy (Black *et al.* 1976).

As energy intake falls, a higher quality of diet is necessary to maintain the same intake of other nutrients. This may be accomplished by the fortification of some foods eaten by adolescents, improved health education, new-product development or by the provision of high-quality foods. The alternative is to increase energy intake by means of increased exercise.

The study was supported by an MRC project grant. The authors thank the Northumberland Health and Education Authorities, and the families involved for their considerable assistance.

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Printed in Great Britain

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ANNALS OF HUMAN BIOLOGY, 1984, VOL. 11, NO. 6, 545–553

A two-year longitudinal study of dietary intake in relation to the growth of 405 English children initially aged 11–12 years

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Received 20 May 1983

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Summary. This report is of a two-year longitudinal study of dietary intake and growth in height and weight of 405 children, initially aged 11–12 years. Between 1979 and 1981 they each recorded their food intake on five occasions for three consecutive days using a diet diary with a 20 min private interview on the fourth day. Computerized food tables were used. Social class was recorded.

The usual differences in height, weight and growth increments between the social classes were found. There were no significant differences in nutrient intake between social classes. Within a social class there were few statistically significant correlations between energy or protein intake and height or weight increments. It was concluded that a very high reliability of food intake measurements would be required in order to relate diet to growth and many other factors (e.g. energy expenditure, body composition and puberty) may also mask any such relationship. Social class alone would seem an inadequate means of allowing for such factors.

1. Introduction

Patton and Findlay (1926), in an M.R.C. report investigating the diet and growth of children, wrote that diet did not seem to be the all-important factor in determining growth. Widdowson (1947) was unable to find a relationship between the energy intakes of children and their height and weight, and made similar comments. However Black, Billewicz and Thomson (1976) found that the energy intakes of preschool children were directly related to their body weight and weight increments. They also found an inverse relationship between energy intake and socio-economic status which has not been found in older children (Cook, Altman, Moore, Topp, Holland and Elliot 1973). Likewise, another study of preschool children (Department of Health and Social Security 1975) reported a similar inverse relationship between social class and nutrient intake, although it was not statistically significant. In spite of their finding that height was related to social class, they also found 'some evidence' of a relationship between energy intake and height. It was concluded therefore that these differences in stature are not necessarily nutritional in origin.

Studies of adolescents indicate different relationships between intake, sex, and social class and stature from those in younger children. Durnin, Lonergan, Good and Ewan (1974) found that only boys (mean age 14·5 yr) from the lowest social class studied had lower nutrient intakes than the other boys, while the intakes of the groups of girls studied were similar. These authors did not relate intake directly to height or weight but they found that the thinnest girls consistently (regardless of social class) ate more than the fattest. However, they reported that the results for the boys were 'confusing'. A study of the intake of primary schoolchildren (10–11 yr) (Darke and Disselduff 1981) again found no relationship between intake and social class, but intake was not compared to height and/or weight. Holland, Rona, Chinn, Altman, Erwig, Cook and Du V Florey (1981) reported that primary schoolchildren (5–11 years) from

manual social classes were smaller than children from non-manual social classes, but geographical region was also found to relate to height: the children from southern England being taller than those from the north. Sibship size was also negatively related to height and it was suggested that economic hardship might account for the relationship with sibship number.

It appears, therefore, that although there is some evidence that the dietary intake of young children is directly related to 'body size' and inversely to social class, these relationships may become less distinct with increasing age. By adolescence there is little evidence of a relationship either between intake and social class or between intake and stature, although there are social class-related differences in stature (Tanner 1976). Two factors may contribute to the inability of some of these studies to demonstrate any relationship between dietary intake and human growth. First, as suggested by Nelson (1980), the quality of the physical and social environment may mediate the effectiveness of diet in promoting growth and health. Secondly, most previous studies have not measured dietary intake over long periods in order to relate it to growth (Hegstead 1976).

This study is the first attempt to relate longitudinal measurements of dietary intake and growth (i.e. height and weight increments) measured over the same period in English adolescents, divided according to their social class.

2. Subjects and methods

This investigation was designed as a longitudinal study relating dental caries increment to diet (Hackett, Rugg-Gunn and Appleton 1983). All the children in their third year at seven of the eight middle schools in the Morpeth, Ashington and Newbiggin area of south Northumberland were invited to take part: one school did not participate because of rebuilding. Informed written consent was obtained from the parents of each child who volunteered. Between September 1979 and October 1981, each child (initially aged 11–12 years) recorded their dietary intake on five separate occasions over three consecutive days. They were asked to record in a specially designed dietary diary all foods and drinks taken. At a private interview on the fourth day quantitative estimates of the foods eaten were made using the child's information and a variety of visual aids (e.g. bowls, cups, spoons and polystyrene shapes of slices of pie or cake) which had been precalibrated with known weights of various foods. These estimates of weights of food consumed were analysed using computerized food composition tables (Paul and Southgate 1978), which were modified and supplemented as required. The method has been fully described elsewhere (Hackett *et al.* 1983). The dietary information given in this report refers to the average diet over the two-year period (mean of 15 days), unless otherwise indicated.

Each child was dentally examined in a purpose-built mobile examining van in October 1979 and October 1981. Height and weight were also measured by the team's radiographer. Height was measured using a sliding headpiece permanently attached inside the van which was maintained level and steady by corner jacks. The children stood without shoes, with their backs against the scale, looking straight ahead, and their height was recorded to the nearest 0·5 cm. Weight was measured to the nearest 0·5 kg using a Seca spring balance, after each child had removed jacket, shoes and any other heavy items of outdoor clothing. No correction was applied to allow for the remainder of the clothing worn (estimated at approximately 0·8 kg per child). The accuracy of the Seca scales was assessed by 10 duplicate weighings, over the range 43–83 kg, comparing the Seca scales with an Avery beam balance. The weighings were

found to be linearly related although the Seca scales consistently underweighed by 1·1%: mean weights (±S.D.) were 48·62 (±27·0)kg and 49·16 (±27·3)kg, respectively. This level of accuracy was considered adequate since analyses were mainly correlation analyses. Quetelet's index (weight/height^2) was calculated for each child at baseline and two years.

The occupation of each child's father was encoded as social class (Registrar General 1970).

Most of the analyses deal with the relationship between an index or child's size or growth rate (height, weight, Quetelet's index or their two-year increment) and a single nutritional factor (energy, protein, fat, CHO or sugar intake) within sex and social class group. In addition to these analyses, several stepwise linear regression analyses were undertaken, again within sexes. In each case, social class and all the nutritional factors were taken as independent variables, but were only allowed to enter the equation after the following had been investigated: dependent variable (i) baseline height no other variable; (ii) baseline weight — baseline height; (iii) height increment — baseline height; (iv) weight increment — baseline weight.

Macronutrient intakes were calculated using a specially written program; most of the rest of the analysis used S.P.S.S.

3. Results

Four-hundred and sixty-six out of 784 children agreed to participate in the survey; the 318 who did not volunteer were not approached further. Of the volunteers, 54 moved away from the area or were absent from one or more dietary surveys, two children asked to leave the study and the data from five children were rejected because of inadequate recording of diet. Thus the results correspond to data from the remaining 405 children (193 boys and 212 girls) who completed all aspects of the study (52% of the target population and 87% of those who volunteered) (table 1). In order to increase the size of the groups, social classes I and II were combined as group 'high', III was labelled 'middle', IV and V as 'low' and VI, VII and VIII as 'miscellaneous'. The number of miscellaneous boys was small (9).

The mean macronutrient and sugars intake of the boys and girls divided according to social class is shown in table 2. There were no statistically significant differences between any of the social classes for any of the dietary variables ($P > 0{\cdot}05$), although the intake of the boys was consistently greater than that of the girls. The nutrient intake

Table 1. Social class distribution of the 405 subjects who completed the study.

			Boys		Girls	
Social class	Description	Group	*N*	%	*N*	%
I		High	7	3·6	13	6·1
II			39	20·2	26	12·3
III		Middle	86	44·6	86	40·6
IV		Low	44	22·8	52	24·5
V			8	4·1	14	6·6
VI	Unclassifiable, unemployed or retired	Miscellaneous	3	1·6	5	2·4
VII			5	2·6	5	2·4
VIII	Unknown		1	0·5	11	5·2
Total			193	100	212	100

Table 2. Daily macronutrient and sugars intake of boys and girls divided according to social class.

		Social class			
		High	Middle	Low	Miscellaneous
Boys					
N		46	86	52	9
Energy	(MJ)	9·4	9·4	9·5	10·7
	(kcal)	2250	2250	2300	2550
Protein	(g)	65	63	64	68
Fat	(g)	102	100	102	109
CHO	(g)	283	286	290	341
Sugars	(g)	127	120	124	143
Girls					
N		39	86	66	21
Energy	(MJ)	8·6	8·3	8·6	8·5
	(kcal)	2050	2000	2050	2050
Protein	(g)	58	54	55	56
Fat	(g)	95	89	95	93
CHO	(g)	256	252	261	256
Sugars	(g)	119	113	111	110

The coefficients of variation of these intakes were very similar between groups and were approximately: energy 15%, protein 17%, fat 18%, carbohydrate 17% and sugars 24%.

Table 3. Mean baseline height and weight and their two-year increments according to sex and social class.

		Social class			
		High	Middle	Low	Miscellaneous
Boys					
N		46	86	52	9
Weight					
Baseline	(kg)	39·9	39·7	37·6	35·3
Increment	(kg)	10·6	10·7	9·2	11·1
Height					
Baseline	(m)	1·48	1·46	1·44	1·44
Increment	(m)	0·14	0·14	0·13	0·14
Girls					
N		39	86	66	21
Weight					
Baseline	(kg)	41·4	38·5	40·8	39·5
Increment	(kg)	9·9	9·2	9·6	10·7
Height					
Baseline	(m)	1·49	1·46	1·47	1·46
Increment	(m)	0·12	0·12	0·11	0·12

The coefficients of variation of these measures were similar between groups and were approximately: baseline weight 20%, weight increment 42%, baseline height 5% and height increment 32%.

has been reported in detail elsewhere (Hackett, Rugg-Gunn, Appleton, Eastoe and Jenkins 1984).

The baseline heights and weights of the children and their two-year increments are shown in table 3. The usual social-class differences were evident although few reached statistical significance in this relatively small sample. All the groups remained on approximately the same percentiles, according to the growth charts of Tanner and Whitehouse (1976).

The Pearson correlation coefficients between height increment and energy or protein intakes, divided according to sex and social class, are shown in table 4. The only statistically significant correlation was between the protein intake of the 'high' social

Table 4. Pearson correlation coefficients between the two-year height or weight increment, final height or weight, and energy or protein intake, according to sex and social class.

			Boys				Girls			
			High	Middle	Low	Miscellaneous	High	Middle	Low	Miscellaneous
N			46	86	52	9	39	86	66	21
Height increment	v.	energy	0·21	0·09	0·07	0·32	0·03	0·16	0·06	0·00
		protein	0·33*	0·09	0·09	0·32	0·12	0·08	0·06	−0·20
Weight increment	v.	energy	0·09	0·10	0·16	0·26	0·06	0·00	−0·02	−0·38
		protein	0·18	0·09	0·25	0·30	0·03	0·11	0·11	−0·33
Final height	v.	energy	0·19	0·19	0·04	0·02	0·48**	0·06	0·24*	0·45*
		protein	0·28	0·18	0·13	0·00	0·46**	0·11	0·30*	0·17
Final weight	v.	energy	0·03	0·03	0·04	−0·18	0·22	−0·05	−0·02	−0·18
		protein	0·16	0·05	0·09	−0·16	0·18	0·07	0·11	−0·13

All correlations were positive unless indicated.
* $P<0·05$, ** $P<0·01$.

class boys and their height increment ($P < 0{\cdot}05$). None of the correlation coefficients between weight increment and energy or protein intake, by sex and social class (table 4), was statistically significant ($P > 0{\cdot}05$).

Combining all the boys together and all the girls together, only the protein intakes of the boys were statistically significantly positively correlated to both their height and weight increments ($r = +0{\cdot}15$, with height increment; $r = +0{\cdot}16$, with weight increment; $N = 193$, $P < 0{\cdot}05$), although both these values are low and explain very little of the variation in height or weight increments.

The correlations between final height and final weight of the children and their mean energy and protein intakes are also given in table 4. Energy intake was significantly positively correlated to final height in only the 'high', 'low' and 'miscellaneous' groups of girls. Protein intake also was positively correlated to the final height of the 'high' and 'low' social class girls.

At age 11·5 yr, 76% of the boys and 70% of the girls had a Quetelet index between 15 and 20. The number of overweight children (Q.I. 25–30), according to Bray (1979), increased from 7 (4 boys, 3 girls) at age 11·5 yr to 15 (6 boys, 9 girls) at age 13·5 yr, while the number of obese children (Q.I. > 30) increased from 2 at age 11·5 yr to 4 at age 13·5 yr. Of the 11 overweight or obese girls, 3 were from the 'miscellaneous' group (whose fathers were either unemployed, retired or of unknown occupation) and 5 from the 'low' social class.

Table 5. Results of stepwise multiple regression analysis with anthropometric measurements as the dependent variable, and anthropometric and dietary measurements, sex and social class as independent variables.

Dependent variable	
Boys	
Baseline height	= 1·450 + 0·0010 protein − 0·017 social class
Baseline weight	= −82·46 + 87·79 baseline height − 0·0545 sugars
Height increment	= −0·168 + 0·209 baseline height
Weight increment	= −7·55 + 0·77 height increment + 0·18 baseline weight
Girls	
Baseline height	= 1·387 + 0·000922 fat
Baseline weight	= −69·26 + 76·43 baseline height − 0·0294 sugars
Height increment	= 0·381 − 0·202 baseline height + 0·00394 energy
Weight increment	= −3·16 + 0·60 height increment + 0·14 baseline weight

Only variables having statistically significant relationships have been included in the equations in this table.

Table 5 lists the stepwise multiple regression equations for each sex using in turn each of the anthropometric variables as the dependent variable. The independent variables were the other anthropometric variables, sex, social class and some dietary factors (energy (kJ), protein (g), fat (g), carbohydrate (g) and total sugars (g) intake per day). No pattern of relationships was observed.

4. Discussion

The increases in height and weight in the present study were not found to be related to dietary intake. These results are in contrast to a study of preschool children which found positive correlations between energy intake and body weight (and weight gain), although the correlations may have decreased in magnitude over the ages eight months

(+0·48) to three years (+0·25) (Black *et al.* 1976). Even within a social class in the present study, energy and protein intakes (or each individuals' two-year increase in energy intake) were found to be not correlated to height or weight increment, except for protein intake and height increment in the 'high' social class boys, which correlation was in any case of a very low order. This might imply that protein intake only affects growth when other factors are favourable but it may be due to chance; there was no such correlation for the 'high' social class girls. However, the girls were likely to have been at a more advanced stage of puberty and were therefore possibly less affected by dietary intake, because their rate of growth was decreasing.

The stepwise multiple regressions show that the influences of the dietary factors (even in combination) are small and not entirely consistent between the sexes. However, many of these variables are intercorrelated, e.g. fat and protein intake ($r = +0{\cdot}78$, $P < 0{\cdot}01$) and the interpretation of such regressions is notoriously difficult. Furthermore, the major determinants of size, e.g. birthweight, parental height, sibship size (Rona, Swan and Altman 1978), have not been measured in this study. Therefore, no particular importance has been attached to the equations produced.

The 'miscellaneous' girls were similar to the 'low' social class girls in several respects, including their tendency to become overweight during the two years. Lower social class adults, especially women, have also been found to be more likely to be obese (James 1980). The prevalence of overweight and 'obesity' in the present study at baseline (2%) was similar to that previously reported in similarly aged Newcastle children (with a different definition of obesity) (Wilkinson, Parkin, Pearson, Philips and Sykes 1977). The prevalence increased slightly, however, from 2% to 4% over the two-year period.

The present study has not resolved the 'anomalies' (the lack of relationship of diet to growth) described by Nelson (1980). There are several possible explanations for this.

First, the estimates of nutrient intake may not have been sufficiently precise to allow a relationship to be demonstrated (Hegstead 1976). For example, the between-social class differences in intake, although not statistically significant, were large enough, if real, to account for large differences in two-year growth. The validity of the method and the reliability of the estimates of energy intake have been reported previously (Hackett *et al.* 1983): the validity was found to be good for groups but poor for individuals, while the reliability of the energy intakes was found to be 0·8. Assuming that each g of tissue gained represents 20 kJ (Payne and Waterlow 1971), it was possible to estimate the energy cost of growth. This was found to average 0·27 MJ/d for the subjects in the present study or only 3% of their average intake. It is not possible to measure an individual's intake to this level of precision at present. Following the method of Payne and Waterlow (1971) further, gave the following partition of energy output: intake 8·95 MJ/d, maintenance 7·50 MJ/d, growth 0·27 MJ/d and 'activity' (by difference) 1·17 MJ/d. The energy available for activity is less than 50% of the amount calculated to be 'available' for children of approximately this age (range 2·26–2·68 MJ/d) by Payne and Waterlow (1971). The assumptions made during calculations of this kind make conclusions at best tentative. Nevertheless, it seems very unlikely that an individual's growth will ever be successfully related to his or her intake due to the relatively small amounts of energy involved in growth.

Secondly, controlling for social class (based on fathers' occupation) alone is not adequate to allow for the environmental factors which affect growth. Thirdly, energy expenditure was not measured in the present study and small daily differences in expenditure could equally account for the lack of relationship between intake and

growth. Fourthly, body composition was also not measured and weight alone is a 'thoroughly unsatisfactory' measure of growth (Tanner 1976). Quetelet's index was calculated but is a measure of the degree of overweight only, as distinct from obesity (Bray 1980). However, Quetelet's index has been shown to correlate with estimates of body fat (Bray 1980). Small differences in the energy density of any tissue gained could mask the effect of diet (or expenditure) on growth. Hackett (1980) found no relationship between usual energy intake and weight in 57 adults, but there was a significant positive correlation with their total body nitrogen and a significant negative correlation with their total body fat content. Finally, no estimates of the stage of puberty were made. Puberty affects different individuals at different times and the various tissues respond differently (Tanner 1981). Hence, there may be changes in the efficiency of energy usage during puberty.

Over the past century there has been a secular trend to increased height of men and women, coupled with a relatively greater improvement in the growth of lower social class children (Tanner 1976). However, the energy intake of children has undoubtedly been falling over this period (Whitehead, Paul and Cole 1982) and this has usually been attributed to a decreased energy expenditure (e.g. Durnin *et al.* 1974). Despite this unpromising background, the lack of a relationship between energy intake and human growth has often been commented upon. The present study has shown that the marked rise in dietary intake of these adolescents was not related in a simple manner to their equally marked growth. A very high degree of reliability of measurement of intake would be required to show a relationship because of the small proportion of intake devoted to growth.

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Zusammenfassung. Dies ist der Bericht einer zweijährigen Längsschnittuntersuchung der Nahrungsaufnahme sowie des Körperhöhen- und Gewichtswachstums von 405 Kindern des ursprünglichen Alters 11 bis 12 Jahre. Zwischen 1979 und 1981 hielt jedes von ihnen die Nahrungsaufnahme zu fünf Zeitpunkten während dreier aufeinanderfolgender Tage mit Hilfe eines Nahrungstagebuches fest, gefolgt von einem privaten Interview von 20 Minuten am vierten Tag. Rechnergestützte Nahrungstabellen wurden benutzt. Die Sozialklasse wurde berichtet.

Die üblichen Unterschiede von Körperhöhe, Gewicht und Wachstumszunahme zwischen den sozialen Klassen wurden gefunden. Es fanden sich keine signifikanten Unterschiede in der Nahrungsaufnahme zwischen den Sozialklassen. Innerhalb einer Sozialklasse gab es wenige statistisch signifikante Korrelationen zwischen Energie- oder Proteinaufnahme und Körperhöhen- oder Gewichtszunahme. Es wurde geschlossen, daß es einer sehr hohen Zuverlässigkeit der Nahrungsaufnahmenmessung bedürfe, um Nahrung mit Wachstum zu verknüpfen, und viele andere Faktoren (z.B. Energieverbrauch, Körperzusammensetzung und Pubertät) können einen solchen Zusammenhang verwischen. Die Sozialklasse allein erscheint als ungeeignetes Mittel, um solche Faktoren einzurechnen.

Résumé. Ce rapport est une étude longitudinale sur deux ans de l'ingestion alimentaire et de la croissance en taille et en poids de 405 enfants, âgés initialement de 11–12 ans. Entre 1979 et 1981 ils ont chacun noté leur ingestion alimentaire à cinq reprises durant trois jours consécutifs en employant un agenda alimentaire, avec entretien privé le quatrième jour. Des tables alimentaires sur ordinateur ont été employées. La classe sociale a été notée.

Les différences habituelles en taille, poids et gains de croissance entre les classes sociales ont été trouvées. Il n'y avait pas de différence significative en ingestion alimentaire entre classes sociales. Dans chaque classe sociale, il y avait peu de corrélations significatives entre l'ingestion d'énergie ou de protéines et les gains de taille ou de poids. Il a été conclu qu'une fidélité très élevée de mesures d'ingestion alimentaire serait nécessaire pour mettre en relation le régime et la croissance, et beaucoup d'autres facteurs (comme la dépense d'énergie, la composition corporelle et la puberté) peuvent masquer une telle relation. La classe sociale seule semblerait un moyen inadéquat de tenir compte de tels facteurs.

David Appleton

Sugars-eating habits of 405 11- to 14-year-old English children

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(*Received 29 June 1983 – Accepted 11 November 1983*)

1. Between September 1979 and July 1981, 405 northern-English children, initially aged 11–12 years, each recorded all food and drink consumed on five occasions on each of three consecutive days. Sugars and nutrient intakes were calculated using food tables.

2. The average total sugars intake was 118 g/d or 21% of the energy intake and 43% of the carbohydrate intake; these percentages were similar for both sexes, all social class groups and surveys.

3. Sugars were found to be derived from a variety of food sources with confectionery being the single largest source.

4. Sugars were fairly evenly consumed over the average day with a high amount in foods eaten between meals. Snacks accounted for 65% of the sugars intake but only 46% of the energy intake.

5. Sugars intake and snacking therefore seem to have been major components of the eating habits of these children. If dietary changes towards lower sugars intakes are to be achieved a vigorous, informed health education campaign is necessary together with effective labelling of manufactured foods, the development of new products and the modification of some existing ones.

A fall in the dietary sugars intake is generally considered to be desirable (United States Senate, 1977; Department of Health and Social Security, 1978; Royal College of Physicians, 1983) mainly because of the improvements in dental health which are likely to result. However, dietary habits are resistant to change (Chambers, 1981) and a thorough knowledge of the eating and social habits of the individual or target population is essential before practical dietary advice can be formulated. It needs to be established which beneficial habits can be encouraged, which habits need to be discouraged, where improvements might be made without entailing major changes to lifestyle and whether a narrow range of foods is responsible for a large proportion of sugars intake. The first comprehensive study of sugars intake was recently published (Morgan & Zabik, 1981) and it supplied some of this information for American children. The aim of this paper therefore is to supply similar information for young English adolescents, who are known to be particularly at risk from dental caries. This was achieved using data obtained from a recent 2-year longitudinal survey of over 400 English adolescents, which had the primary aim of relating estimates of individuals' mean dietary intake to their observed dental caries increment.

METHODS

Between September 1979 and July 1981, over 400 children initially aged 11–12 years recorded their entire dietary intake for three consecutive days on five separate occasions. These children were volunteers from seven schools in the Morpeth, Ashington and Newbiggin area of Northumberland. Each child was asked to record, in a specially designed diary, a description of all foods and drinks taken, the approximate amount consumed in household measures and the time of consumption. They were then individually interviewed on the 4th day when the information provided was verified and enlarged upon. Using the child's description and a variety of calibrated visual aids, a quantitative estimate of intakes

* For reprints.

was obtained. A note was also made of the combinations of food items consumed simultaneously (labelled 'foods' in this survey) which were aggregated to give the weight of foods consumed per day. Computerized food tables (Paul & Southgate, 1978) were used to calculate the weight of total sugars and nutrients consumed, and the methods have been fully described elsewhere (Hackett *et al.* 1983). These food tables discriminate only lactose from all the other sugars which are totalled together. The term total sugars or sugars in this paper refers to the total of all sugars consumed (including lactose). Table sugar refers to sucrose as used in the home. Further analysis of the types of sugars consumed has not been attempted.

In order to aid the location of the sources of the dietary sugars, the foods were grouped together as follows: (1) confectionery, which included boiled sweets, pastilles, gums, chewing gum, toffees, popcorn, iced-lollies, ice cream, pure chocolate bars, filled chocolates (and bars) and full coated chocolate biscuits; (2) table sugar which consisted almost entirely of refined white sucrose but included the various 'brown' sugars; (3) the soft drinks category which included carbonated and still drinks, e.g. lemonade, cola, cordial and fruit juice but not tea, coffee or milk shakes; (4) lactose which was the total of the sugar lactose consumed in whatever form; (5) the biscuits and cakes category (flour and sugar products) which included all sweet (but not savoury) biscuits, half-coated chocolate biscuits and cakes, including those coated with chocolate; (6) puddings which included milk puddings (e.g. rice), sweet sauces, yoghurts, all flour-based puddings (e.g. pies), tinned and stewed fruits and jellies; (7) fruit which consisted mainly of raw fresh fruit, as dried fruit was excluded from all groups except where it occurred in recipes; (8) the syrups and preserves group which was composed of jam, marmalade, honey, treacle, syrup, lemon curd, marzipan and mincemeat.

The foods consumed were also categorized according to their sugars concentration: a food was defined as the sum of those items of diet (as listed in the food tables) which were consumed simultaneously (e.g. tea plus milk plus sugar or, bread plus butter plus boiled egg). This provides an estimate of the concentration of sugars actually reaching the mouth.

The occupation of each child's father was encoded as social class (Registrar General, 1970). In order to increase the size of the groups analysed, classes I and II were grouped together and labelled 'high' (professional occupations), III was labelled 'middle' (skilled workers) and IV and V 'low' (manual workers). Classes VI, VII and VIII (retired or unemployed, unknown or unclassifiable occupations) were labelled 'miscellaneous', although only small numbers were involved (nine boys, twenty-one girls).

All the information collected was stored in a computer and all analyses were done either using purpose-written programs or, in the case of standard statistical methods (*t* test or analysis of variance), using Statistical Package for the Social Sciences (SPSS).

RESULTS

All aspects of the study were completed by 193 boys and 212 girls or 51·7% of those asked to volunteer. The three groupings were well represented with 85 'high', 172 'middle' and 118 'low' social class children. Only one out of the 405 subjects in the study was non-caucasian.

The average daily intakes at each of the five surveys for boys and girls are shown in Table 1. The boys, who increased their energy intake by about 13% over the 2 years (calculated by regression), consistently consumed more energy than the girls, who increased their intake by only about 7%. The carbohydrate and sugars intake changed in proportion to the energy intake; carbohydrate supplied about 49% and sugars about 21% of the energy intake. Of the carbohydrate, sugars accounted for 43% for both boys and girls.

There were no statistically significant differences in energy, carbohydrate or sugars intake

Table 1. *Mean daily intake of energy, carbohydrate and total sugars for 193 boys and 212 girls at each of the five surveys*

(The coefficients of variation were similar for both sexes at each survey and were approximately: energy 22%, carbohydrate 23%, total sugars 33%)

Survey...	1	2	3	4	5	Mean
Boys						
Energy (MJ)	8·9	8·9	9·3	10·0	10·2	9·5
Carbohydrate (g)	274	269	285	300	317	289
Total sugars (g)	121	118	122	126	133	124
Girls						
Energy (MJ)	8·1	8·4	8·3	8·7	8·8	8·5
Carbohydrate (g)	249	254	252	257	268	256
Total sugars (g)	114	116	109	111	116	113

(weight or percentage) between social classes within each sex (Table 2). The 10% of subjects (plus 'ties', *n*42) with the highest sugars intake (> 159·5 g/d) had a higher energy intake than for all subjects and a higher percentage of energy from sugars (25), and of carbohydrate as sugars (49), but only a slightly higher percentage of energy from carbohydrate (51). Twenty-seven of these forty-two subjects were boys, but the social classes were all well represented (ten, seventeen, eight and seven for 'high', 'middle', 'low' and 'miscellaneous' respectively).

The very wide range in mean sugars intake can be seen in Fig. 1, which gives the frequency distribution of daily sugars intake averaged over all 15 d.

There are some small differences in the sources of the sugars intake between sexes and social classes (Table 3). The girls derived less sugars from table sugar and lactose, but a higher percentage from confectionery, soft drinks, biscuits, cakes and fruit than the boys. The 'low' social class boys and girls derived a higher percentage of their sugars intake from confectionery and table sugar but a lower percentage from soft drinks compared with 'high' or 'middle' social class boys and girls. The high sugars eaters showed no preferences for the form in which the sugars were consumed compared with all the children.

For all subjects, only 24% of the total weight of foods consumed had a total sugars concentration of 1% or less (Table 4) and 19% of the total weight of foods consumed had a sugars content of over 10%. For both boys and girls there was a significant decrease in the percentage of foods containing over 10% sugars, as social class became lower (boys: $F_{1,181} = 5{\cdot}38$, $P < 0{\cdot}05$; girls: $F_{1,188} = 8{\cdot}43$, $P < 0{\cdot}01$). For boys only, there was also a significant decrease in the percentage of foods containing less than 1% sugars as social class became lower ($F_{1,181} = 5{\cdot}08$, $P < 0{\cdot}05$). The proportion of foods consumed of more than 60% sugars was very low. In the high sugars eaters, a lower proportion (20%) of the foods were of less than 1% sugars content compared with the other children ($t_{60} = 3.82$, $P < 0{\cdot}001$) and more of their foods were in the over 10% sugars category ($t_{47} = 5{\cdot}30$, $P < 0{\cdot}001$). These t values were calculated by Welch's adaptation of the t test (Bailey, 1981).

Fig. 2 shows the weight of sugars consumed for each half-hour of the day averaged over all 15 d for all 405 subjects. There were clearly four peak intakes (roughly 07.00–09.00, 12.00–13.00, 16.00–18.00, 20.00–22.00 hours) corresponding to breakfast, lunch, evening meal (tea) and supper. These periods were then used to compile Table 5 which summarizes, on average, the times when sugars were eaten. The evening meal (16.00–18.00 hours) accounted for the highest percentage of daily sugars intake (21.5%), and 48·4% of all sugars were consumed after 16.00 hours. The children's average bedtime was about 22.00 hours

Table 2. *Daily intake of energy, carbohydrate (CHO) and total sugars according to sex, social class and sugars intake*
(Mean values and standard deviations)

Social class	*n*	Energy (MJ)		CHO (g)			Total sugars (g)			
		Mean	SD	Mean	SD	% of energy	Mean	SD	% of energy	% of CHO
Boys										
High	46	9·4	1·3	283	43	48	127	28	22	45
Middle	86	9·4	1·5	286	47	49	120	27	20	42
Low	52	9·5	1·4	290	45	49	124	25	21	43
Girls										
High	39	8·6	1·5	256	50	48	119	31	22	46
Middle	86	8·3	1·3	252	46	49	113	28	22	45
Low	66	8·6	1·5	261	51	48	111	32	21	42
High sugars eaters*	42	10·9	1·2	350	35	51	171	11	25	49
All subjects	405	8·9	1·5	272	51	49	118	29	21	43

For all comparisons between social classes within a sex, $P > 0{\cdot}05$ (analysis of variance).
* 10% of subjects (plus 'ties') with the highest sugars intake (> 159·5 g/d).

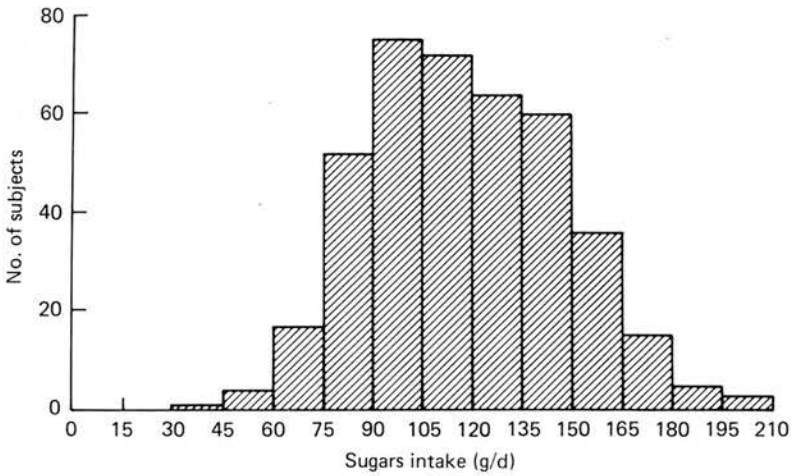

Fig. 1. Frequency distribution of sugars intake/d for 405 children.

Table 3. *Mean daily total sugars intake (%) from each of eight food categories*

Sex...	Boys			Girls				
Social class...	High	Middle	Low	High	Middle	Low	High sugars eaters*	All subjects
n...	(46)	(86)	(52)	(39)	(86)	(66)	(42)	(405)
Food category								
Confectionery	13	16	19	15	17	22	20	19
Table sugars	14	18	21	11	16	17	17	17
Soft drinks	14	14	11	17	16	11	14	14
Lactose	15	13	13	12	12	11	12	13
Biscuits and cakes	13	12	12	14	13	14	12	13
Puddings	10	10	8	12	9	9	10	11
Fruit	4	3	4	6	5	5	4	5
Syrups and preserves	6	4	3	3	3	3	4	4
Breakfast cereals	3	3	2	3	2	2	2	2
Other foods	8	7	7	7	7	6	5	2

* 10% of subjects (plus 'ties') with the highest sugars intake (> 159·5 g/d).

and very few children recorded intakes after going to bed. The lunch time (12.00–13.00 hours) peak is remarkable for its brevity whilst the breakfast (07.00–09.00 hours) peak indicates that breakfast is still a popular meal. A consistent 2–3% of sugars intake was taken after 22.00 hours (suggesting the consumption of sugars close to bedtime in a substantial number of children) but a very low percentage was consumed before 07.00 hours. Most sugars were consumed as snack intakes rather than as meals (Table 6). The snack intakes were poor sources of protein and the boys and girls consumed almost identical amounts of energy as snacks; the boys therefore ate larger meals.

Because a large number of subjects was studied, it is possible that some of the smaller differences between groups, although statistically significant, are of little practical importance. Furthermore, the large number of tests performed demands that caution be applied when interpreting the results.

Table 4. *Foods* (% by weight) consumed/d according to their sugars content†*

Social class	*n*	Mean daily weight of all foods consumed (g)	Sugars concentration of foods			
			⩽ 1·0%	1·1–10·0%	10·1–60·0%	> 60·0%
Boys						
High	46	1801	24	55	20	1
Middle	86	1789	24	57	18	1
Low	52	1781	20	62	17	1
Girls						
High	39	1684	28	51	20	1
Middle	86	1633	25	57	17	1
Low	66	1642	25	58	15	2
High sugars eaters‡	42	2090	20	56	22	2
All subjects	405	1713	24	57	18	1

* A food is defined as any combination of items (as listed in food tables) consumed simultaneously, e.g. tea infusion plus milk plus sucrose is one food.

† The standard deviations were similar between the groups and were approximately: total weight of all foods, 287; ⩽ 1·0% sugars, 10; 1·1–10·0% sugars, 11; 10·1–60·0% sugars, 7; > 60·0% sugars, 0·9.

‡ 10% of subjects (plus 'ties') with the highest sugars intake (> 159·5 g/d).

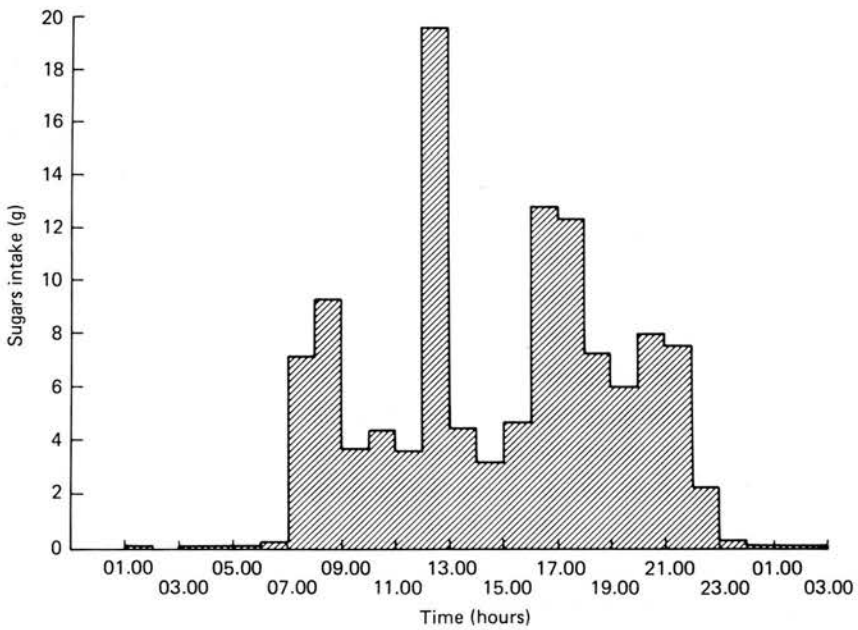

Fig. 2. Mean weight of sugars consumed for each hour of the day.

DISCUSSION

The diary-and-interview method proved an acceptable means of recording the dietary habits of a large number of adolescents over a long period of time. Five surveys of three consecutive days during a 2-year period gave, by analysis of variance (Goldstein, 1979), a reliability coefficient of 0·78 for estimating sugars intake enabling high and low sugars eaters to be differentiated (Hackett *et al.* 1983).

Table 5. *Mean sugars intake (%) consumed by time of day according to sex, social class and total sugars intake*

Time (hours)... Social class	*n*	Before 07.00	07.00–09.00	09.00–12.00	12.00–13.00	13.00–16.00	16.00–18.00	18.00–20.00	20.00–22.00	After 22.00
Boys										
High	46	0·3	15·3	9·4	16·7	9·0	21·7	12·5	13·0	2·1
Middle	86	0·2	14·4	10·3	16·5	10·2	21·3	10·8	13·6	2·6
Low	52	0·2	15·6	9·8	15·9	9·8	21·5	9·4	16·4	2·8
Girls										
High	39	0·3	12·0	10·1	18·6	11·1	21·1	12·7	11·8	1·9
Middle	86	0·3	13·8	10·6	16·6	12·2	21·0	11·2	12·1	2·1
Low	66	0·3	11·7	9·9	15·6	10·7	23·8	11·8	13·5	2·5
High sugars eaters*	42	0·3	13·5	9·7	14·3	11·5	22·3	10·7	14·5	3·0
All subjects	405	0·3	14·0	10·1	16·6	10·6	21·5	11·2	13·3	2·4

* 10% of subjects ('plus ties') with the highest sugars intake (> 159·5 g/d).

Table 6. *Mean daily intake (%) of macronutrients and total sugars consumed as snacks*, according to sex*

	Boys	Girls
Energy	42 (4·0 MJ)	46 (3·9 MJ)
Protein	34	37
Fat	38	43
Carbohydrate	47	52
Sugars	62	65

* Defined as an intake having at least one of the following properties: (*a*) thought to make a minor contribution to the day's intake, (*b*) eaten whilst engaged in some other activity, (*c*) not eaten at recognized mealtimes or places.

Unfortunately, most recent British dietary surveys of adolescents do not report sugars intake in great detail. Cook *et al.* (1973) reported 'added sugar' intake only, which supplied approximately 18% of the energy intake of their sample of children aged 8–15 years (boys 113 and girls 90 g sugars/d). Likewise 'added sugars' was the only category reported by Darke & Disselduff (1981) and supplied about 16% (boys 89 and girls 80 g sugars/d) of the energy intake of 10–11-year-olds from three English cities. The study of Widdowson (1947) carried out between 1935 and 1939 gives figures which apparently are for added sucrose only. The 11-, 12- and 13-year-old boys averaged 112 and the girls 89 g sucrose/d or 17 and 15% of their energy intakes respectively. Because it is unlikely that sugars occurring naturally have different cariogenicities than added sugars, the proportion of sugars added during processing and preparation were not calculated in the present study. The findings of the above studies (notwithstanding the differences in reporting sugars intake) are broadly in line with the present study suggesting that, over-all, the contribution of sugars intake has changed little over these years. Energy intakes, however, have changed markedly (Whitehead *et al.* 1982) and the type of sugars consumed and the way in which they are used have also changed (Bibby, 1975). Energy intake seems to be the major factor which determines the weight of sugars consumed. Although reliability was the same, the coefficient of variation of sugars intake was higher than that for energy, indicating that sugars intake was more variable, between children, than energy intake. Sugars intake was not strongly related to social class but there was a very small fall in the contribution of sugars to energy intake with increasing age (Table 1).

Durnin *et al.* (1957) reported the intake of twelve young Glaswegian women, mean age 20·5 years: sugar and preserves supplied 8·1% of their energy intake and sweets and chocolate 6·2%, both figures very much higher than for the younger girls of the present study (4·4 and 4·0% respectively). No figures for total sugars intake were reported. These differences could be due to the differences in the age or locality of the subjects or to a change in habits over the last 25 years or to a combination of these factors.

The only investigation comparable to the present study is that conducted by Morgan & Zabik (1981). They published an analysis of the sources of sugars intake of American children aged 5–12 years (total sugars intake for 11–12-year-olds 143 g/d). There are some striking differences between their findings and those of the present survey. Their largest source of sugars was from milk, which contributed 20% of the total, but in the present study only 13% of the sugars was lactose. Confectionery was the major component of the sugars intake of the English children (19%) whereas the American children consumed only 8% of their sugars as candy plus sweet dairy products and frozen confectionery (a roughly analogous grouping). The Americans consumed over twice as much sugars as fruit (11%) as the English (5%), and more as sweetened beverages (23%, including fruit juices,

compared with 14%). Table sugar contributed less than 10% of the Americans' sugars intake but 17% of the English children's sugars intake. Although there were differences between the two surveys, both sets of eating habits were associated with a wide range of foods containing sugars and with high total sugars intake, and both surveys suggest frequent snacking.

The 25% of American children with the highest sugars intake consumed their sugars in various forms, but the groups of dessert sauces, fruits and fruit juices, and cakes, cookies, pies and other desserts, were judged to have the greatest impact, although the maximum difference was less than 3%. The 10% of subjects with the highest sugars intake in the British survey did seem to have a slightly 'sweet tooth', consuming sugars over and above their high energy intakes. Unlike the American children, however, they did not show any preference for the form in which the sugars were consumed. Sugars were widely distributed throughout their diet, several (arbitrary) food categories contributing large proportions of the total and no one category contributing the major proportion. The sugars intakes were also widely spread throughout the day, although there was a preference for consuming sugars during the late afternoon and evening (48%) and between main meals (34·6%) (Table 5). These observations suggest that no one group of foods can be held responsible for high sugars consumption (although some individuals may have particular likes).

Most of the sugars intake (62%) was consumed in the form of snacks. This is possibly a growing, relatively modern habit and is dentally undesirable (Report to the Nutrition Foundation, 1980). Dietary habits (the manner in which, as well as which particular foods are eaten) are clearly changing in line with changes in other behaviour patterns: working mothers, less formal intra-family relationships, greater independence of children, etc., and these factors should be considered when attempts are made to change dietary habits. Snacking is probably here to stay (Truswell & Darnton-Hill, 1981); there is therefore a need for safe snacks to be developed. The criteria which define a safe snack are beyond the scope of this paper but should include foods of low sugars content.

Sugars were found to be a major dietary component, thoroughly dispersed throughout the diet and day's eating. Sugars are presently an integral part of the trend towards smaller, more frequent snack intakes and are introduced at a very early age (King, 1978). Even with ill-health (e.g. coronary heart disease) as a warning, dietary changes can be difficult to achieve, food intake being inexorably combined with other aspects of behaviour, habits and personality. If half of the soft drinks consumed were sweetened only artificially, sugars consumption would fall by approximately 7%; if confectionery intake were halved, sugars intake would fall by about 10%; if table sugar usage were halved, total sugars intake would decrease by approximately 8% and if sweet biscuit and cake consumption were halved, sugars intake would fall by 6%. All these measures are realistic. If all were implemented, assuming that the foods substituted were sugars-free (it is possible that the consumption of some sugared foods, e.g. jam, would increase slightly), sugars intake could fall by 25–30% and a marked improvement in the figures for dental caries would be expected to occur. It is suggested that such changes would be assisted by the development of safe snacks, the modification of the composition of some existing products (particularly soft drinks and confectionery), effective food labelling and more health education campaigns.

This research was supported by an M.R.C. Project Grant.

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Printed in Great Britain

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Sugars Consumption of Northumbrian Children Aged 11–14 Years

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ABSTRACT

In order to investigate the relationships between diet and tooth decay the authors recently completed the first longitudinal study of diet and dental caries increment in children. 405 children initially aged 11.5 years each recorded their intake of foods and drinks for a total of 15 days over a period of 2 years. This is a review of some of the findings.

The evidence implicating consumption of sugars in the causation of dental caries is both diverse and overwhelming (Rugg-Gunn, 1983). Of the 30 or so observational studies relating diet and dental caries in children, all except one have been cross-sectional in design. Such a design is unsatisfactory in older children at least because there is little reason why a lifetime's caries experience should be related to a single estimate of dietary intake. In addition there have been few reports of the food intake in general, and sugars intake in particular, of children in the UK. A longitudinal survey of intake could indicate how choice of foods changes with age and if repeated could show how preferences change over time. The National Food Survey has provided some information on food acquisition (not consumption) over the past 40 years (Derry and Buss, 1984). Unfortunately, besides not estimating consumption this survey does not include foods bought and consumed away from the home such as confectionery and soft drinks. Furthermore, this survey collects data by household and so information for age and sex specific groups is not available. Comments regarding sugars consumption amongst children must be derived from data collected from properly designed dietary surveys of defined groups of individuals. Such knowledge of what children eat is essential to the planning of effective health education campaigns.

In order to contribute to the understanding of the relationship between diet and caries in children and to contribute to health education a longitudinal study was undertaken which had the following aim: to rank some defined dietary factors either singly or in combination in the order in which they explain the dental caries increment of over 400 children.

METHODS

In July 1979 all the third year children, initially aged 11–12 years, attending seven middle schools in the Morpeth, Ashington and Newbiggin area of south Northumberland were asked to volunteer. This area was chosen because it is outside the fluoridated water zone of Newcastle and it gives a range of social classes. Each child was dentally examined (including bite-wing radiographs) during October 1979, 1980 and 1981, in a mobile examining van. Their heights

and weights were also measured at these times and a report of their growth in relation to diet has been published (Hackett et al, 1984c).

On five occasions between September 1979 and July 1981, each child recorded all foods and drinks consumed in a specially designed diary for three consecutive days. The children also recorded their times of consumption, estimates in household measures of how much was eaten, whether each item was consumed alone or in combination with any other item and their bedtimes. An account of their bedtimes has also been published (Rugg-Gunn et al, 1984a). Each child was then interviewed on the fourth day by one of us (AFH) to verify and enlarge upon the information produced and an arbitrary assessment was made of whether each intake constituted a meal or snack. By questioning and with the aid of food models a quantitative estimate of intake was arrived at (Hackett et al, 1983).

Computerised food tables were used to convert the weights of food consumed to nutrient intake and total sugars intake (Paul and Southgate, 1978). By modifying the food tables an estimate of the amount of sugars judged to have been added by food manufacturers, cook or consumer was also arrived at. In order to locate the dietary sources of the sugars intake the foods were grouped together into various arbitrary categories e.g. confectionery (Sweets, chocolates, ice cream and full coated chocolate biscuits).

Foods were also categorised according to their sugars concentration: a food was defined as those items (as listed in the food tables) consumed simultaneously e.g. tea plus milk plus sugar.

The occupation of the fathers of the children were recorded and coded as social class. Groups I and II were then labelled "high", III as "middle", and IV and V as "low". Groups VI (unclassifiable) VII (retired or unemployed) and VIII (unknown) were generally excluded from any analyses by social class due to the small numbers involved.

RESULTS

All aspects of the study were completed by 193 boys and 212 girls or 52% of those asked to take part. The social classes were well represented with 85 "high", 172 "middle" and 118 "lower" social class children. Only one child in the study was non-caucasian. These proportions were similar to those expected for this area at that time, but the sample cannot necessarily be considered to be a representative one.

The average daily intakes for the boys and girls are shown in table 1 and have been reported in more detail (Hackett et al, 1984a). The boys, who increased their

TABLE 1
MEAN DAILY ENERGY, CARBOHYDRATE AND SUGARS INTAKE BY SURVEY AND SEX

Survey	1		2		3		4		5	
Sex	M	F	M	F	M	F	M	F	M	F
Energy (MJ)	8.9	8.1	8.9	8.4	9.3	8.3	10.0	8.7	10.3	8.8
CHO (g)	274	249	269	254	285	252	300	257	317	268
Sugars (g)	121	114	118	116	122	109	126	111	133	116
CHO/energy (%)	49	49	48	48	49	49	48	47	49	49
Sugars/energy (%)	22	23	21	22	21	21	20	20	21	21
Sugars/CHO (%)	44	46	44	46	43	43	42	43	42	43

The coefficients of variation were virtually the same for each sex at each survey: energy 22%, carbohydrate 23% and sugars 33%.

TABLE 2
MEAN DAILY INTAKE OF TOTAL SUGARS AND ADDED SUGARS FROM VARIOUS FOOD CATEGORIES AS A PERCENTAGE OF THE TOTAL

Food category	Total sugars (%)	Added sugars (%)
Confectionery	19	28
Table sugars	17	24
Soft drinks	14	17
Lactose	13	00
Biscuits and cakes	13	12
Puddings	11	9
Fruit	5	0
Syrups and preserves	4	3
Breakfast cereals	2	3
Other foods	2	3

TABLE 3
MEANS AND 95 PER CENT LIMITS FOR WEIGHT AND FREQUENCY OF INTAKE OF SOME DIETARY VARIABLES

Dietary variable	Weight (g)		Frequency (per day)	
	Mean	limits	Mean	limits
Sweets	22	2- 61	1.0	0.1- 2.6
Chocolate	35	6- 80	0.9	0.1- 2.1
Puddings	75	8-107	0.5	0.1- 1.2
Soft drinks	60	0-189	0.7	0.0- 2.1
Milk	250	52-595	2.3	0.5- 4.0
Apples	25	0- 82	0.3	0.0- 0.9
Other fruits + raw vegetables	30	0- 96	0.5	0.0- 1.3
All foods and drinks	1713	1201-2336	6.8	4.3-11.7
Foods >1.0 % sugars	1301	746-1950	6.0	3.9-10.0
Foods >10.0 % sugars	324	138- 618	3.7	1.7- 6.4
Foods >60.0 % sugars	22	2- 57	1.0	0.1- 2.7

energy intakes by about 13% over the two years, consistently ate more than the girls, who increased their energy intakes by only 7%. The carbohydrate (CHO) and sugars intake changed in proportion to the energy intake although the possibility of a slight fall in the contribution of sugars over the two years can be seen. The overall mean sugars intake was 118 g per day (or 21% of the energy) but there was a very wide range in the sugars intake between children (coefficient of variation 33%). The sugars intake has also been reported in detail elsewhere (Hackett et al, 1984b). There were no statistically significant differences in energy, CHO or sugars intake as weight or percentage between the social classes within each sex.

The mean added sugars intake was 81 g per day or 69% of all sugars consumed or 15% of the energy intake; this proportion was similar between the sexes and social classes. Snacks were found to contribute 44% of the energy intake, 63% of the total sugars, 66% of the added sugars but only 34% of the protein. This information has also been previously reported (Rugg-Gunn et al, 1986).

The origin of the sugars and added sugars intake is shown in table 2. Although no single group of foods contributed by far the largest proportion of the total

sugars (or added sugars) consumed, the large combined contribution of confectionery, table sugars and soft drinks is notable. Conversely, the minor contributions of fruit, preserves, breakfast cereals as such and miscellaneous items such as baked beans (part of the "other" foods) can also be seen.

The weight and frequency of eating of several food groups is shown in table 3. The mean confectionery intake was 57 g per day and 1.8 of the 6.8 intakes per day included or consisted entirely of confectionary. Only 24% of the total weight of all foods and drinks consumed (which included water, meat etc.) had a sugars content of less than 1%. However, 19% of all foods by weight had a sugars content of more than 10%. In addition 54% of the food intakes contained at least one food of 10% or more sugars content.

DISCUSSION

The methodology of this study has been discussed at length elsewhere together with the dental findings: (Rugg-Gunn et al, 1984; Hackett et al, 1985).

Sugars are not an essential dietary component. This study has clearly shown that sugars were nevertheless a major component of the diet and daily eating pattern of these children. Sugars were mainly added by the food manufacturers, cook or consumer and only about a third were those which occurred naturally in the foods eaten. It is the added sugars component which should be the focus of health education campaigns partly because they made up the largest component of the sugars intake and partly because the naturally occurring sugars were consumed in foods having other desirable dietary properties (e.g. vitamins, minerals and fibre). The results of this study also showed that between two thirds and three quarters of the added sugars were from confectionery, soft drinks and table sugar. This clearly suggests that it is these three groups that should be highlighted in health education campaigns, rather than such minor contributors to added sugars consumption as baked beans, jam and marmalade and breakfast cereals as such. Furthermore, these latter foods would be eaten at, and not between, meals which would be expected to be less damaging to the teeth due to the protective influence of the saliva flow and other dietary components (Rugg-Gunn, 1983).

The energy intake of children appears to have been falling over the last 3–4 decades (Whitehead et al. 1982). If children are to maintain their intakes of essential nutrients they will need to consume a diet of increasing nutrient density (g nutrients per MJ). This in turn suggests that the foods to eat less of are those which contribute only energy to the diet. Table sugar (sucrose) and soft drinks are two of the very few such foods commonly eaten by children. These two foods were found to contribute 6.2% of the energy intake of these children (Hackett et al, 1986).

These children averaged nearly seven intakes of food per day, many of which were snacks rich in added sugars, and frequent eating of sugary foods is considered to be the habit most conducive of caries. Snacking, however, is here to stay (Truswell and Darnton-Hill, 1981), and there is therefore the need for "safe" snacks to be developed which should include low sugars content (natural or otherwise).

Desirable changes in eating habits may come about largely because an individual decides, perhaps in response to a health education campaign, to alter his or her habits, this requires knowledge and information. Knowledge amongst children, parents and teachers about the role of sugars in the causation of dental caries is very good (Ndobe, unpublished 1986). Sadly, however, many foods which have high sugars content are not being labelled as such.

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Nutrition and Health, 1987, Vol. 5, No. 1/2, pp. 19–23.

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Human Nutrition: Applied Nutrition (1986) **40A**, 115-124.

THE DIETARY INTAKE OF ADDED AND NATURAL SUGARS IN 405 ENGLISH ADOLESCENTS

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A number of reports have urged a reduction in sugars intake. Implementing this advice depends upon knowledge of sugars intake by target groups. This paper reports the added and natural sugars intake by one target group — young adolescents. The diet of 405 children was recorded using a 3-day diary with interview repeated five times between September 1979 and July 1981. Food tables (Paul & Southgate, 1978) were used and the values for the sugars content of the food items altered to provide both added sugars and natural sugars concentration. The mean daily intake of added and natural sugars respectively, for the 193 boys was 85 g (s.d. 22), 39 g (s.d. 12), and for the 212 girls was 78 g (s.d. 24), 35 g (s.d. 12). Added sugars contributed, on average, 69 per cent of total sugars and 15 per cent of energy intake. Confectionery, table sugar and soft drinks together contained 71 per cent of the added sugars intake, while milk, fruit and their products provided the majority of the natural sugars. It is considered that major reductions of sugars intake would be possible in these children by restricting the intake of a small number of groups of foods of low nutrient density. Food labelling of sugars content would enable consumers to make sensible choices of foods.

Introduction

It is desirable to have information on the consumption of sugars, first, because sugars are the major dietary cause of dental caries and, second, because foods with a high sugars content tend to be low in other nutrients. With the energy intake of people living in the UK declining (Buss, 1979; Whitehead, Paul & Cole, 1982) it would seem necessary that the nutrient density of our diet is high.

Although the prevalence of dental caries in children in the UK has declined in recent years (Todd & Dodd 1985), its cost in financial and social terms is still considerable. It is important, therefore, that effort continues to be made to prevent this disease in which diet has an important aetiological role, and information on sugar consumption is essential basic knowledge to enable health education to achieve this goal.

Most surveys have recorded only 'added' sugars and only a few have recorded 'total' sugars consumption, which can be defined as the sum of both 'natural' and 'added' sugars. One reason for this is that information on the total sugars content of all foods has only been widely available in the UK since the publication of the fourth edition of *McCance and Widdowson's 'The Composition of Foods'* (Paul & Southgate, 1978). The third edition (McCance & Widdowson, 1960) gave sugar content but excluded some natural sugars (e.g. lactose in milk). Knowledge of the levels and patterns of consumption of total sugars is valuable in nutritional research, while knowledge of the levels and patterns of consumption of added sugars is of particular assistance in health education. The purpose of the present article is to provide

information on the consumption of natural and added sugars, and the sources of these sugars, in a group of English adolescents. Information on the total sugars consumption by these subjects has been published previously (Hackett *et al.*, 1984*b*).

Subjects and methods

The design and conduct of the study has been described by Hackett *et al.* (1983, 1985). Briefly, between September 1979 and July 1981, over 400 children living in south Northumberland and initially aged 11-12 years recorded their diet for three consecutive days on five occasions. At each occasion each child was interviewed on the fourth day by a nutritionist to clarify details in the child's diary and to convert the amounts consumed from household measures to grams weight. The occupation of each child's father was coded as social class (Registrar General, 1970). All dietary and social data were entered into computer files and, using computerized food tables (Paul & Southgate, 1978), the weights and frequencies of intake of dietary variables were obtained for each child at each survey.

In these food tables, the sugars content of each food item was given as the total sugar content, i.e. all free mono- and di-saccharides. In the present analyses, the sugars columns in these tables were altered to give the added sugars content of each item. 'Added sugars' were defined as 'any sugars added during the processing or preparation of a food by a manufacturer, cook or consumer'. The difference between the original total sugars value and the substituted added sugars value was then taken to be the natural sugars content of each food. The added sugars values were determined for each food item, in order of preference, by: (1) inspection of recipes, the sources of recipes being *McCance & Widdowson's 'The Composition of Foods'* (Paul & Southgate, 1978), *Practical Cookery* (Ceserani & Kinton, 1974) and *The Times Cookery Book* (Stewart, 1975); (2) manufacturers' information; and (3) dissection of foods which consisted of several components (e.g. fruit pies, chocolate and biscuit snacks): dissection indicated the proportion by weight of the components. Other sources of information consulted were Department of Health and Social Security Food Tables (unpublished) and Southgate *et al.* (1978).

The foods consumed were categorized according to their sugars concentration: a food was defined as the sum of those items of diet (as listed in the food tables) which were consumed simultaneously (e.g. tea plus milk plus sugar). This provides an estimate of the concentration of sugars actually reaching the mouth.

In order to identify the sources of the added and natural sugars in the diet, the foods were grouped together as follows: (1) confectionery, which included boiled sweets, pastilles, gums, toffees, popcorn, iced-lollies, ice-cream, pure chocolate bars, filled chocolate and bars, and fully-coated chocolate biscuits; (2) table sugar, which consisted almost entirely of refined white sucrose, but included various brown sugars; (3) soft drinks, which included carbonated and still drinks, e.g. lemonade, cola, cordial and fruit juice, but not tea, coffee, chocolate or milk shakes; (4) biscuits and cakes

(flour and sugar products) which included all sweet (but not savoury) biscuits, part-coated chocolate biscuits and cakes; (5) sweet puddings, which included milk puddings, sweet sauces, yoghurts, all flour-based puddings (e.g. pies), tinned and stewed fruits, and jellies; (6) syrups and preserves, which included jam, marmalade, honey, treacle, syrup, lemon curd, marzipan and mincemeat; (7) breakfast cereals, which included corn- and rice-based flakes and grains, porridge and muesli-type mixtures; (8) milk, cheese and butter, which included fresh whole, sterilized, longlife, skimmed and goat's milk, single, double, whipping and sterilized canned cream, matured, processed, cottage and cream cheeses and cheese spreads, butter, margarine and low-fat spreads, but excluded milk, cheese or butter included in recipes; and (9) fruit, which included raw fresh fruit and dried fruit except where it occurred in recipes.

Each dietary intake for each child was classed as either a meal or snack: a snack was defined as an intake having at least one of the following properties: (1) it was thought to make a minor contribution to the day's intake, (2) it was eaten whilst engaged in some other activity, (3) it was not eaten at recognized meal-times or places.

Results

Four hundred and sixty-six children (59 per cent of those invited) volunteered to take part in the study. Of these, 193 boys and 212 girls completed all aspects of the 2-year study. Their mean age in October 1979 was 11 years 7 months. The percentages of children in each social class group were: I + II 21 per cent, III 43 per cent and IV + V 29 per cent, with 7 per cent miscellaneous (social classes VI to VIII).

Out of the 999 codes in the expanded food tables, 636 were used by any of the 405 subjects on any of the 15 days recorded by each subject; 206 of these 636 codes contained added sugars. Inspection of recipes and packages was sufficient to determine the added sugars content of nearly all items, but food dissection was necessary in a few cases.

The proportion of the total sugars that was added sugars remained almost constant over the 2 years: 68 to 70 per cent both in the boys and in the girls. Likewise the proportion of carbohydrate that was added sugars differed little, at 29 to 30 per cent for the boys and 29 to 32 per cent for the girls, between the five surveys despite a rise in energy, carbohydrate and total sugars intake over the 2 years, particularly in the boys (Hackett *et al.*, 1984*a*).

Between social classes within each sex, differences in the proportions of total sugars that were added sugars were observed (Table 1). In neither sex were these differences statistically significant ($P > 0.05$), but for the boys the percentages were 65 per cent for social classes I + II, 69 per cent for III and 71 per cent for IV + V; for the girls the corresponding percentages were very similar at 65, 68 and 71 per cent. There was little difference in the proportions of added sugars in the carbohydrate or energy intakes between social classes in either sex. For all 405 subjects, the mean added sugars intake was 81.3 g/day (s.d. 23.6) and the mean natural sugars intake was 37.1 g/day (s.d. 11.9); added sugars contributed 69 per cent of the total sugars intake,

30 per cent of the carbohydrate and 15 per cent of the energy intake. Differences can be seen in Table 1 between the 41 (10 per cent) subjects with the highest added sugars intake and the 41 subjects with the lowest added sugars intake. Added sugars contributed nearly one-fifth of the energy intake of the children with a high added sugars intake, but only one-tenth of the energy intake of the children with a low added sugars intake. The proportions of energy from natural sugars in the high and low added sugars-eaters were similar (6 and 7 per cent, respectively).

Table 1. *Mean daily intake of energy, carbohydrate, total and added sugars according to sex and social class and for the 10 per cent of subjects with the highest and 10 per cent of subjects with the lowest added sugars intakes*

	n	*Energy* (*MJ*)	*Carbo-hydrate* (*g*)	*Total sugars* (*g*)	*Added sugars*				
		mean (s.d.)	*mean (s.d.)*	*mean (s.d.)*	*mean* (*g*)	*(s.d.)*	*% of tot. sugars*	*% of CHO*	*% of energy*
Boys									
I + I	46	9.4 (1.3)	283 (43)	127 (28)	82.8	(22.4)	65	29	14
III	86	9.4 (1.5)	286 (47)	120 (27)	83.1	(20.7)	69	29	14
IV + V	52	9.5 (1.4)	290 (45)	124 (25)	87.5	(21.2)	71	30	15
Girls									
I + II	39	8.6 (1.5)	256 (50)	119 (31)	76.8	(23.9)	65	30	14
III	86	8.3 (1.3)	252 (46)	113 (28)	77.1	(22.0)	68	31	15
IV + V	66	8.6 (1.5)	261 (51)	111 (32)	78.7	(22.9)	71	30	15
All subjects	405	8.9 (1.5)	272 (51)	118 (29)	81.3	(23.6)	69	30	15
High-added-sugar eaters	41	10.8 (1.2)	349 (34)	169 (14)	126.2	(13.4)	75	36	19
Low-added-sugar eaters	41	7.6 (1.4)	214 (38)	77 (14)	45.3	(6.9)	59	21	10

The consumption of added sugars from various dietary sources is given in Table 2 and 3, as grams weight and as a percentage of the total added sugars intake. Data, corresponding to Table 2, for natural sugars, are given in Table 4. For all subjects 23.0 g of added sugar was consumed in confectionery and 19.6 g as table sugar, on average per day (Table 2). These two dietary sources contributed 52 per cent of the mean daily intake of added sugars. If the 13.6 g of added sugars from soft drinks is included, these three sources contributed 69 per cent of the mean daily intake of added sugars. Only 2.7 g (3 per cent) of added sugars was unaccounted for by the groups of foods listed in Table 2.

Some differences between social classes were observed (Table 2). Whereas for confectionery and table sugar, consumption of added sugars was higher in social classes IV + V compared to social classes I + II ($P < 0.05$ for both boys and girls), the reverse social class trend was seen for soft drinks (statistically significantly different in the girls only, $P < 0.01$) and for syrups and preserves (boys, $P < 0.001$; girls, $P < 0.05$).

The 10 per cent of subjects with the highest added sugars intake consumed, on average, 126 g of added sugars per day (Table 3); nearly three

Table 2 *Mean daily intake of added sugars from various dietary sources (as g and as percentage of added sugars intake), according to sex and social class. Standard deviations given for all subjects only*

Dietary source		*Boys*			*Girls*			
		I + II (n = 46)	*III* (n = 86)	*IV + V* (n = 52)	*I + II* (n = 39)	*III* (n = 86)	*IV + V* (n = 66)	*All subjects* (n = 405)
Confectionery	g	19.6	21.3	25.6	20.3	22.0	26.3	23.0 (± 13.2)
	%	24	26	29	26	28	33	28
Table sugar	g	17.9	21.3	25.6	12.9	17.6	18.9	19.6 (± 12.5)
	%	22	26	29	17	23	24	24
Soft drinks	g	15.0	14.4	12.0	16.5	15.3	10.6	13.6 (± 9.4)
	%	18	17	14	21	20	13	17
Biscuits & cakes	g	10.0	10.1	10.1	10.8	9.2	10.5	10.1 (± 6.0)
	%	12	12	12	14	12	13	12
Sweet puddings	g	8.4	7.4	6.4	9.0	6.2	6.5	7.2 (± 4.5)
	%	10	9	7	12	8	8	9
Syrups & preserves	g	5.4	3.3	2.1	2.7	2.4	1.8	2.8 (± 3.2)
	%	7	4	2	4	3	2	3
Breakfast cereals	g	3.8	2.4	2.6	2.4	2.1	1.5	2.3 (± 3.0)
	%	5	3	3	3	3	2	3
Other	g	2.7	2.9	3.3	2.2	2.4	2.7	2.7 (± 3.1)
	%	3	3	4	3	3	3	3
All sources	g	82.8	83.1	87.5	76.8	77.2	78.7	81.3 (± 23.6)
	%	100	100	100	100	100	100	100

times the intake of the 10 per cent of subjects with the lowest intake of added sugars. While 58 per cent of the daily added sugars intake in the high sugar eaters came from confectionery and table sugar, 47 per cent came from

Table 3. *Mean daily intake of added sugars from various dietary sources (as g and as percentage of added sugars intake) for the subjects with lowest and highest added sugars intake*

		Lowest 10% of added sugars eaters (< 53.5 g/day) (n = 41)	*Highest 10% of added sugars eaters (> 112.0 g/day)* (n = 41)
Confectionery	g	11.8	40.3
	%	26	32
Table sugar	g	9.7	32.2
	%	21	26
Soft drinks	g	7.2	20.8
	%	16	16
Biscuits & cakes	g	6.3	14.5
	%	14	11
Sweet puddings	g	5.4	9.1
	%	12	7
Syrups & preserves	g	1.7	3.6
	%	4	3
Breakfast cereals	g	1.0	2.4
	%	2	2
Other	g	2.2	3.2
	%	5	3
All sources	g	45.3	126.2
	%	100	100
Energy (MJ)		7.56	10.81

confectionery and table sugar in the low-sugar eaters ($P < 0.01$). The reverse trend was seen for biscuits, cakes and sweet puddings where the percentage contribution of these sources to the daily intake of added sugars was 18 per cent for the high-sugar eaters and 26 per cent for the low-sugar eaters ($P < 0.001$) (Table 3).

One-third of the daily intake of natural sugars in all 405 subjects was provided by milk, and 15 per cent by fruit (Table 4). Sweet puddings, which contain milk and fruit, also contributed 15 per cent of the natural sugars intake. Children from classes I + II consumed more natural sugars than social classes IV + V children ($P < 0.01$) (Table 4) despite similar energy intakes (Table 1). As far as the dietary sources of the natural sugars were concerned (Table 4), differences between social classes were less marked than those observed for added sugars (Table 2) and were inconsistent between the sexes.

Table 4. *Mean daily intake of natural sugars from various dietary sources (as g and as percentage of natural sugars intake), according to sex and social class. Standard deviations given for all subjects only*

		Boys			*Girls*			
Dietary source		*I + II* ($n = 46$)	*III* ($n = 86$)	*IV + V* ($n = 52$)	*I + II* ($n = 39$)	*III* ($n = 86$)	*IV + V* ($n = 66$)	*All subjects* ($n = 405$)
Confectionery	g	1.4	1.3	1.4	1.4	1.4	1.3	1.3 (± 0.9)
	%	3	3	4	3	4	4	4
Soft drinks	g	2.7	2.3	1.8	3.8	2.9	1.7	2.4 (± 2.4)
	%	6	6	5	9	8	5	6
Biscuits & cakes	g	3.4	2.8	2.2	3.8	2.7	2.8	2.9 (± 2.4)
	%	8	7	6	9	8	9	8
Sweet puddings	g	6.0	5.9	4.6	7.0	4.7	4.7	5.5 (± 3.7)
	%	14	16	13	17	13	14	15
Syrups & preserves	g	2.6	1.7	1.6	1.3	1.1	1.3	1.5 (± 1.7)
	%	6	5	4	3	3	4	4
Breakfast cereals	g	0.6	0.7	0.5	0.7	0.5	0.3	0.5 (± 1.1)
	%	1	2	1	2	1	1	1
Milk	g	16.2	12.4	13.2	10.8	11.0	9.3	11.8 (± 6.4)
	%	37	33	36	26	31	29	32
Fruit	g	4.9	4.4	5.4	7.3	6.1	6.1	5.5 (± 4.4)
	%	11	12	15	17	17	19	15
Other	g	6.0	5.9	5.6	5.7	5.6	5.0	5.7 (± 5.0)
	%	14	16	15	14	16	15	15
All sources	g	43.8	37.4	36.3	41.8	36.0	32.5	37.1 (±11.9)
	%	100	100	100	100	100	100	100

The contribution of added sugars to the total sugars content of foods differed between the various dietary sources (Tables 2 and 4). Ninety-five per cent of the sugars content of confectionery was added sugars, while this was 85 per cent for soft drinks, 82 per cent for breakfast cereals, 78 per cent for biscuits and cakes, 64 per cent for syrups and preserves and 57 per cent for sweet puddings.

Sixty-three per cent (74.8 g) of all sugars was consumed in snacks (Table 5). A bigger proportion of added sugars was consumed in snacks (66 per cent, 53.6 g), compared with the proportion of the natural sugars intake that

was consumed in snacks (57 per cent, 21.2 g). Expressing the results in Table 5 another way, 72 per cent (53.6 g) of the sugars in snacks was added sugars, while 64 per cent (27.7 g) of the sugars in meals was added sugars.

The 405 subjects consumed a mean of 1.713 kg of food (and drinks) per day. Forty-three per cent, by weight, of this intake contained 1.0 per cent or less of added sugars. Forty-six per cent of the daily food intake contained between 1.1 and 10.0 per cent added sugars, 10 per cent of food contained 10.1 to 60.0 per cent added sugars, while 1 per cent contained over 60.0 per cent added sugars.

Table 5. *The mean (± s.d.) weights (g) of natural and added sugars that were consumed in snacks or meals in 405 adolescents*

Sugars	*Snack*	*Meal*	*All intakes*
Natural	21.2 (7.8)	15.9 (7.5)	37.1 (11.9)
Added	53.6 (21.1)	27.7 (12.0)	81.3 (23.6)
Total	74.8 (24.8)	43.6 (18.0)	118.4 (29.4)

Discussion

The method used to estimate the dietary intake of these adolescents has been discussed previously (Hackett *et al.*, 1983, 1985) and reports of some aspects of the nutrient intake of these children have been published (Hackett *et al.*, 1984*a*,*b*,*c*). The increase in energy intake during the 2 year study has also been commented upon previously (Hackett *et al.*, 1983, 1985) and reports of some aspects of the nutrient intake of these children have been published (Hackett *et al.*, 1984*a*) and is in agreement with increasing energy intake during adolescence reported by Nelson, Dyson & Paul (1985). The data collected would appear to be valid and reliable. The groupings of foods are similar to those used in a previous publication (Hackett *et al.*, 1984*b*). The definition of 'added' sugars is simple and logical but the interpretation of this definition could be controversial for a small number of food items. In this study, all sugars (McCance & Widdowson codes 841 to 845) as well as honey (code 848) were classed as added sugars, since their primary purpose was for sweetening and their nutrient value other than energy was negligible. On the other hand, sugars in dried fruit were classed as natural sugars so that, for example, mincemeat (code 855) was estimated to contain 48.0 per cent natural sugars and muesli (code 50) was estimated to contain 16.2 per cent natural sugars.

There have been few reports of sugars intake for specific age groups in the UK to compare with the findings of this study. It is unique in reporting natural, added and total sugars intake. Only Morgan & Zabik (1981) have published information on the total sugars intake of children, and our findings have already been compared with this American survey (Hackett *et al.*, 1984*b*). Recently, the total sugars intake of Royal Navy submariners has been published (Gilbert & Brooks, 1985): although the mean intake of these young male adults was very high at more than 166 g sugars/day, this provided only 14 per cent of their energy intake, which is a lower percentage than recorded in our study (21 per cent).

Cook *et al.* (1973) reported the added sugars intake of 8- to 15-year-old children in Kent as: boys 113 g sugars/day, girls 90 g sugars/day, or approximately 17 per cent of their energy intake. The corresponding weights in our study were: boys 85 g/day, girls 78 g/day, or 15 per cent of their energy intake. Our figures are very similar to those reported by Darke & Disselduff (1981) for 10- to 11-year-old children in three English cities: boys 95 g sugars/day and girls 85 g sugars/day, or about 16 per cent of energy intake. Whether these three sets of data indicate a secular change in added sugars intake is unclear as there are so few data for specific age groups. Black, Ravenscroft & Sims (1984) have recently reported that the added sugars intake of 42 dietitians was 38 g sugars/day; 11 of them had intakes of less than 25 g sugars/day. These weights were much lower than those published by Bingham, McNeil & Cummings (1981) for Cambridgeshire men (91 g sugars/day) or women (57 g sugars/day). On a national basis, for all age groups combined, the refined sugar moving into human consumption in the UK in 1980 (the middle of our study) was 37.8 kg/person/year (104 g/person/day) (Ministry of Agriculture, Fisheries and Food, 1983), which is considerably higher than the 81.3 g of added sugars per day consumed by our 11- to 14-year-old children. In 1980, 'consumption' of 45 g of sugar/person/day was recorded by the UK household food consumption and expenditure survey (Ministry of Agriculture, Fisheries and Food, 1982): this figure is difficult to compare with our data because this figure refers to sugar bought as such and foods high in sugars were excluded from the survey.

The main finding of our study was that, on average, 69 per cent of sugars intake was added sugars and only 31 per cent natural sugars. A high proportion of the added sugars occurred in confectionery, table sugar, biscuits and cakes which are foods of low nutrient density, while the natural sugars were an integral part of milk, fruit, and their products which, in turn, are more desirable components of our diet. It would seem sensible, therefore, to urge a reduction in the added sugars, rather than the natural sugars component; this could result in a maximum reduction of 69 per cent of all sugars in these children. However, this maximum is very unlikely to be obtained (Black *et al.*, 1984). How age-specific these findings are is unknown.

The information on the dietary sources of added sugars is useful in health education. It shows, *inter alia*, the unimportance of sources such as ketchup and baked beans, even in these adolescents, since, on average, only 3 per cent of added sugars did not come from the seven groups of foods listed in Table 2. Emphasis should be to reduce consumption of confectionery and table sugar and to reduce the sugars content of soft drinks. This agrees with Black *et al.* (1984) who urged that education should be specifically directed at reducing the intake of confectionery, mineral drinks and sugars in beverages, in order to reduce sugars consumption.

There are three main approaches to reducing sugars consumption in the community: (a) by advice to either individuals or groups, (b) by legislation (e.g. control of advertising sugary products to children, or the introduction of informative food labelling), and (c) by the substitution of sugars by alternative, non-cariogenic sweeteners (Rugg-Gunn & Edgar, 1985). All of

these approaches would benefit from accurate information on sugars consumption and the sources of added sugars. Although there is evidence that children are becoming more knowledgeable concerning the dangers of high sugars consumption (Cresswell *et al.*, 1983; Todd & Dodd, 1985) it would seem that food labelling of sugar content is an essential step in reducing sugar consumption.

Acknowledgements — This study was supported by an MRC Project Grant.

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Caries Research
Editor: Klaus G. König, Nijmegen

David Appleton

Reprint
Publisher: S. Karger AG, Basel
Printed in Switzerland

Caries Res. *18:* 149–152 (1984)

© 1984 S. Karger AG, Basel
0008–6568/84/0182–0149 $ 2.75/0

Correlations of Dietary Intakes of Calcium, Phosphorus and Ca/P Ratio with Caries Data in Children[1]

(Short Communication)

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Key Words. Calcium · Caries, human · Diet · Phosphate

Stanton [1969] reported that caries activity in 183 adult patients was strongly related to the calcium-phosphorus (Ca/P) ratio in their diet estimated from records kept by the patients for 1 week. 80 of the patients were classified as caries inactive: the mean Ca/P ratio of their diet was 0.57. The 103 patients who were classified as caries susceptible (but without quoting precise caries scores or increments) were divisible into two groups – 45 with a high Ca/P ratio (mean 0.79) and 58 with a low Ca/P ratio (mean 0.39). The differences between the dietary Ca/P ratio of the caries inactive patients and the Ca/P ratios of the two caries susceptible groups were statistically highly significant ($p < 0.001$ for each comparison). *Stanton* [1969] suggested that the dietary Ca/P ratio influenced the enzymic activity of oral bacteria and stated that alteration of an unfavourable Ca/P ratio in the diets of caries active patients to a favourable Ca/P ratio (0.57) resulted in the successful control of caries activity, although no details were given. Because *Stanton*'s [1969] report on the effect of dietary Ca/P ratio on caries development in human subjects is unique and indicated a strong correlation, this relationship has been investigated in adolescent English children.

405 children, initially aged 11.5 years, took part in a 2-year observational study into the relation between diet and caries. Only 1 child was non-Caucasian. The dietary information was obtained using a 3-day record with a 20-min interview on the 4th day. This was repeated five times during the 2-year study period, resulting in a total of 15 days of dietary record for each child. All dietary data were collected by 1 dietitian.

Food quantities were recorded by the child in household measures and converted to gram weight at the interview with the aid of food models. The composition of the diet of each child was calculated from the food tables of *Paul and Southgate* [1978] as the mean of the values obtained on the 15 days. Each child received 3 an-

[1] Supported in part by the Medical Research Council Project Grant G978/746/SB.

Table I. Daily dietary intake of Ca, P and Ca/P ratio, together with baseline DMFS and 2 year DMFS increment (Δ), for 193 boys and 212 girls aged 11.5–13.5 years

	Ca mg	P mg	Ca/P	DMFS	
				baseline	Δ
Boys, mean ± SD	891 ± 220	1,124 ± 206	0.79 ± 0.08	4.74 ± 3.76	3.81 ± 3.69
90% range	608 – 1,180	830 – 1,395	0.68 – 0.89	0 – 10	0 – 9
Girls, mean ± SD	775 ± 193	978 ± 191	0.79 ± 0.09	5.16 ± 4.01	3.46 ± 3.48
90% range	530 – 1,025	732 – 1,220	0.68 – 0.90	0 – 11	0 – 8

Table II. Pearson's correlation coefficients between daily dietary intake of Ca, P or Ca/P ratio, and baseline DMFS or 2-year DMFS increment, in 193 boys and 212 girls aged 11.5–13.5 years

	Ca	P	Ca/P
DMFS (baseline)			
Boys	+0.000	+0.029	−0.050
Girls	−0.003	+0.007	−0.019
DMFS (Δ)			
Boys	−0.065	−0.049	−0.063
Girls	+0.088	+0.126	−0.025

Data transformed to correct for skewness. $p > 0.05$ for all correlations.

nual caries examinations (including bitewing radiographs) over the 2-year study period using the method of *Rugg-Gunn* et al. [1973], except that partial recording was used [*Rugg-Gunn* et al., 1975]. The partial recording method (96 sites/mouth) includes 95% of the baseline DMFS at the age of 11.5 years and 96% of the subsequent 2-year increment.

The same examiner was employed throughout the study. Precavitation carious lesions were excluded since the cavitation grade is the stage at which a surface requires restoration [*Downer*, 1975].

The mean daily energy intake was 9,475 kJ for the 193 boys and 8,468 kJ for the 212 girls. This agrees with other recent British studies of this age group [*Darke* et al., 1980] and indicates that the recording of the energy level of the dietary intake was accurate. The mean daily Ca and P intakes were higher in the boys than in the girls but the mean Ca/P ratios were the same (0.79; table I). The range of Ca/P ratios in the 405 children was 0.56–1.04, slightly greater in the girls than in the boys and much narrower than that recorded by *Stanton* [1969] (0.24–1.05). The baseline DMFS and 2-year DMFS increment for boys and girls (table I) are similar to caries levels reported in recent British studies [*Anderson* et al., 1982].

The absence of any subjects with the low dietary Ca/P ratios that *Stanton* [1969] associated with caries activity makes it impossible to test his conclusion that both high and very low ratios favoured caries. To correct the skewed distributions of the variables, the data were transformed in the following ways: Ca and P, log (x); DMFS (baseline), log (x + 0.5)

and DMFS (Δ), log (x + 1.5). The correlations on the transformed data were little different from those obtained from the original data and none was statistically significant. All the correlations between the three dietary variables and DMFS (table II) were low and statistically non-significant. Similarly, none was significant ($p > 0.1$) when data for boys and girls were combined (data not given in tables). If anything, our results showed a trend toward a negative correlation between caries experience or increment and Ca/P ratio. The 23 subjects (5% + equalities) with the lowest Ca/P ratios (0.56–0.66, equivalent to the intermediate ratios which *Stanton* [1969] found were associated with the absence of caries activity) had a mean baseline DMFS of 5.17 and a 2-year DMFS increment of 3.35. The 22 subjects (5% + equalities) with the highest Ca/P ratios (0.93–1.04) had a mean baseline DMFS of 4.55 and a 2-year DMFS increment of 4.23. The differences in caries scores between the children with a high Ca/P ratio and those with a low Ca/P ratio were not statistically significant for either baseline ($p > 0.4$) or incremental ($p > 0.4$) data, thus not supporting *Stanton*'s [1969] conclusion. No correlation was observed between caries and dietary Ca and P intakes (table II).

There are some methodological differences between this study and that of *Stanton* [1969]. While the present study was restricted to 11.5- to 13.5-year-old children, *Stanton*'s [1969] study was on adults (although age was not reported). Patients in *Stanton*'s [1969] study kept a 7-day diet record and approximately 25% of them weighed all foods, although the mixing of dietary survey methods is inadvisable [*Young and Trulson*, 1960] as different methods may cause different eating patterns to be followed or recorded. In the present study a 15-day total unweighed record was obtained. As food tables are a major source of error in this type of survey [*Woolf*, 1954] weighing is unlikely to have increased the accuracy of the dietary measurements. *Stanton* [1969] did not divide his data according to his subject's age, race and sex, factors which invariably affect food intake. Caries activity 'was estimated by examining dental histories and the present oral conditions' with examinations every 6 months while, in the present study, caries examinations were standardised and increments recorded.

A great many studies of the influence of the dietary calcium and phosphorus levels on dental caries have been conducted in animals. Those concerned with dietary Ca/P ratios are mainly irrelevant to the human situation either because they involve both upper and lower extremes of Ca/P ratios [*Sobel* et al., 1960], or use desalivated animals which were fed diets supplemented with phosphate [*Wynn* et al., 1956]. Such supplemented diets (e.g. with added sodium phosphate) are unlikely to mimic self-selected diets in human subjects (with the extra phosphorus coming largely from protein) with regard to the availability of phosphorus in the mouth.

It is clear that *Stanton* [1969] was interested in a post-eruptive dietary effect since adults were his subjects. While the present study does not confirm the existence of a post-eruptive influence of the Ca/P ratio in diet, the pre-eruptive effect of changes in the dietary Ca/P ratio has yet to be investigated in human subjects.

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The importance of fortification of flour with calcium and the sources of Ca in the diet of 375 English adolescents

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(*Received 13 May 1983 – Accepted 19 September 1983*)

1. The contribution of the fortification of flour with calcium carbonate to the Ca intake of 375, 11–14-year-old Northumbrian children has been calculated.

2. Mean Ca intakes were above those currently recommended for children of this age. The fortification of flour supplied 16% of the total Ca intakes.

3. Without the fortification, the percentage of these children with intakes below the recommended intake would be more than double. This would include the majority of girls (particularly those of social classes III, IV and V).

4. The recommendation to stop fortifying flour with calcium carbonate should be reviewed.

With the progressive increase in life expectancy (Anon., 1982) osteoporosis and its consequences in the elderly are making increasing demands upon National Health Service resources. Any measures therefore, including nutritional ones, which may help reduce the incidence of such bone disease merit very serious consideration.

Bone is progressively lost from the skeleton from the fourth decade onwards (Robinson, 1983) and the major factors which determine the susceptibility of an individual to fracture are the rate of bone loss and the mass of bone existing at the onset of bone loss (Newton–John & Morgan, 1968). There is evidence, in caucasian subjects at least (Mathovic *et al.* 1979), that an inadequate dietary intake leads to a significantly reduced bone mineral mass at maturity. It is particularly during the adolescent growth spurt with its increased calcium requirement (Garn, 1981) that the dietary supply of Ca may become limiting. Estimates of requirement at this important stage of development are as high as 1·4 g/d (Albanese, 1977) and if this is not satisfied, a lower skeletal mass at maturity may result with the attendant consequences in terms of fracture liability in later life.

Since 1942, all wheat flour except wholemeal has been fortified with calcium carbonate, which at present contributes between 0·94–1·54 g Ca/kg flour (Department of Health and Social Security, 1981*a*) and is a significant source of dietary Ca. The reasons for this addition were: the likely scarcity of milk and cheese and the increase in the proportion of bran in flour for bread-making during wartime, the desire to maintain the Ca intakes of infants following the lowering of the vitamin D content of some foods and, finally, the possibility that a relatively high Ca intake may protect against cardiovascular disease (Department of Health and Social Security, 1981*a*). The expert panel of the Committe on Medical Aspects of Food Policy considered these reasons no longer valid (Department of Health and Social Security, 1981*a*). They also stated that there was no evidence that the removal of the fortification would cause the intake of population groups to fall below the current recommendations.

This paper reports a recent survey of the Ca intake of a group of adolescents at the time of their growth spurt, in South Northumberland, calculated both with and without the contribution from the fortification.

* For reprints.

Table 1. *Average calcium intake, by sex and social class, calculated with and without the contribution from fortification, and percentage of subjects below current recommendations**

(Mean values and standard deviations)

Sex	Social class†	*n*	Daily Ca intake (mg)		Daily Ca intake (mg/MJ)		Daily Ca intake (mg) less fortification		Fortification as percentage of total intake	Percentage of subjects below recommended intake	
			Mean	SD	Mean	SD	Mean	SD		With fortification	Without fortification
Boys	I+II	46	953	229	101	19	815	216	15	13	35
	III	86	864	235	92	18	715	228	17	26	52
	IV+V	52	871	186	92	9	731	184	16	17	40
Girls	I+II	39	836	211	98	9	706	197	15	28	54
	III	86	780	197	94	9	659	186	16	34	64
	IV+V	66	742	179	87	5	615	158	17	42	71

* Boys and girls (11–14 years) = 700 mg/d (Department of Health and Social Security, 1979).

† Registrar General (1970).

Table 2. *The percentage contribution (and weight) of the three largest contributing groups of food (milk, fortified flour and cheese) to the total daily calcium intake of 11–14-year-old children, by sex and social class*

		Social class*					
		I+II		III		IV+V	
Food	Sex	%	mg	%	mg	%	mg
Milk	M	53	502	48	411	49	424
	F	43	363	46	36	42	315
Fortified flour†	M	16	155	19	167	18	158
	F	18	147	17	135	19	143
Cheese	M	7	67	6	55	5	47
	F	11	95	9	69	6	42

* Registrar General (1970).
† Includes the small amount of Ca which occurs naturally.

SUBJECTS AND METHODS

Between September 1979 and July 1981, 405 children from seven South Northumberland middle schools each completed five dietary records of three consecutive days (15 d total record per child) (Hackett *et al.* 1983). The children were initially aged 11–12 years and were asked to record all foods and drinks taken, indicating the amount consumed in household measures. On the 4th day all the children were individually interviewed, by the same nutritionist, to verify and enlarge upon the recorded information which was converted to Ca intake using food tables (Paul & Southgate, 1978). The total daily Ca intake per child, including water, and the weight and percentage of Ca from various groups of foods, were calculated. Foods containing wholemeal flour and self-raising flour were grouped separately from foods containing other flour, since wholemeal flour is not fortified with Ca and self-raising flour is fortified to a variable extent depending upon the raising agent used. By modifying the food tables, the weight of Ca consumed which originated from the fortification of flour (which was mandatory throughout the survey) was calculated: a figure of 1·35 g Ca/kg fortified white flour (Paul & Southgate, 1978) was used and allowance was made for all other Ca sources in each recipe as appropriate. The occupation of each child's father was coded as social class (Registrar General, 1970).

RESULTS

Only 375 out of the 405 children were classified into social classes I to V, and further analyses were limited to these children. The mean daily Ca intake ranged from 953 mg/d for boys in the social classes I+II to 742 mg/d for girls in the social classes IV+V (Table 1). The boys' Ca intake was higher than the girls' ($P < 0{\cdot}01$) and, for both sexes, social classes I+II had higher intakes than social classes IV+V ($P < 0{\cdot}05$). Although the total energy intakes of boys were consistently higher than those of girls, no difference was observed between social classes ($P > 0{\cdot}05$). Differences were, however, observed in the daily Ca intake per unit of energy between social classes, with the boys in the social classes I+II having higher intakes than those in social classes III, IV and V ($P < 0{\cdot}05$), and girls in the social classes IV+V having lower intakes than those in social classes I+II and III ($P < 0{\cdot}05$). The percentage of the total daily Ca intake due to fortification was similar between the groups and ranged from 15 to 17%. Without the fortification, the percentage of children with daily Ca intakes

less than the recommended level (700 mg Ca (Department of Health and Social Security, 1979)) more than doubled. This increase was from 13 to 35% for the boys in the social classes I+II, but was more marked (from 42 to 71%) for the girls in the social classes IV+V. The mean Ca intake of the girls of social classes III, IV and V would have fallen below the recommendation.

Milk, fortified flour and cheese provided nearly three-quarters of the total daily Ca intake for these children (Table 2). Other foods (e.g. wholemeal and self-raising flour, confectionery, potatoes, eggs, soups, baked beans, meat and fish, green vegetables and breakfast cereals) were found, individually, to contribute only a small proportion of the total daily Ca intake. Milk provided nearly half the Ca intake. No differences between sexes and social classes in the percentage contribution of fortified flour (including the naturally occurring Ca) to daily Ca intake were observed. Small differences between sexes and social classes were observed in the contribution of cheese to the total daily Ca intake, with girls in the social classes I+II (11% or 95 mg/d) having twice the intake of those of the social classes IV+V (6% or 42 mg/d) ($P < 0{\cdot}001$).

DISCUSSION

Although milk intake has been falling for several decades (Angel & Hurdle, 1978), the results from the present survey confirm that milk is still the major dietary source of Ca. The nutritional significance of the reduction in the milk intake is somewhat reduced by the fact that cheese intake has risen progressively since the end of food rationing (Angel & Hurdle, 1978). Flour, however, is the second most important source, but the Ca in white flour is largely (90%) the result of fortification. The Ca intakes recorded in the present survey were similar to those reported in several other surveys of adolescents (Darke *et al.* 1980; Darke & Disselduff, 1981). However, none of these studies considered the contribution of the fortification of flour to Ca intake.

This study shows that even with the modest dietary Ca requirement of 700 mg/d for children of 11–14 years, some 13–26% of male subjects and 28–42% of female subjects fall beneath the recommended level. Furthermore, much higher Ca intakes have been recommended (Albanese, 1977; Marcus, 1982). More importantly, the study also shows that removal of the Ca added to flour (which comprised about 16% of Ca intake) would increase the proportion falling below the recommended intake to between 35 and 40% of males and 54 and 71% of females. Removing the Ca fortification from white flour would significantly reduce the mean dietary Ca intakes of the groups of children studied. Contrary to the views of the expert panel, the mean Ca intakes of an important population group (the girls of social classes III, IV and V) would fall below current recommendations.

The panel have argued that there was no evidence of Ca deficiency in countries where Ca intakes were 'considerably less' than in Britain, implying that present recommendations may be too high. However, the studies on low Ca intakes in other countries (Nordin, 1976) were carried out on populations of non-caucasian extraction, usually negro, and it is known (Nordin, 1976; Garn, 1981) that their bone metabolism is significantly different from that of caucasians and such extrapolations may therefore be invalid. For such studies to be meaningful they should be true longitudinal studies.

The energy intake of children has been falling for several decades (Whitehead *et al.* 1982), therefore there is a need for the consumption of a diet of higher quality (nutrient/MJ) if requirements are still to be met. Fortification is perhaps one way of achieving this. This is particularly true of the lower social class girls who consume diets of the poorest quality (mg Ca/MJ).

The consequence of the inadequate Ca intake at this critical stage of skeletal development is likely to be less dense bone on maturity, with the prospect of an increased likelihood of

skeletal fractures in later life. One of the preferred choices (Robinson, 1983) in the treatment of demineralizing disorders, such as osteoporosis, is supplemental Ca; vitamin D and its metabolites, and anabolic sex hormones being the other major therapeutic agents employed. Since, particularly in the elderly, such treatment with Ca may be effective and inhibit (if not reverse) the progressive loss of bone, reducing the dietary supply of Ca, by removing the fortification of flour, may require increased prescribing of expensive Ca supplements. However, Stevenson & Whitehead (1982) have suggested that Ca supplements alone give only small benefits, although these authors emphasise the importance of a diet adequate in Ca. Stevenson & Whitehead (1982) also describe the cost (£48 million in 1981) of fracture of the neck of the femur, which they suggest is largely due to post-menopausal osteoporosis. A proportion of these costs may be saved by maintaining the fortification of flour with Ca.

Implementation of the expert panel's recommendations not to fortify flour would lead to a 16% reduction in Ca intake; this appears to be contrary to the current emphasis on preventive medicine (Department of Health and Social Security, 1981*b*). Therefore it is suggested that this recommendation is re-examined.

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Printed in Great Britain

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Human Nutrition: Applied Nutrition (1986) **40A,** 176-184

DIETARY SOURCES OF ENERGY, PROTEIN, FAT AND FIBRE IN 375 ENGLISH ADOLESCENTS

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The aim of this report of the dietary sources of energy, protein, fat and fibre in 375 English adolescents aged 11-14 years is to contribute to the development of health education. Each child recorded their dietary intake five times over a 2-year period using a 3-day diary with interview. Food tables (Paul & Southgate, 1978) were used to calculate nutrient intake. The results are the mean of all 15 days intake. Potatoes (chips and crisps) were the largest single source of energy but 'meat' was the main source of protein and fat, other important sources of fat were the spreading fats, milk, chips and crisps. Chips were the main source of fibre but white bread, crisps and baked beans also contributed similar proportions of fibre. Some differences between the sexes and social classes were observed; the lower social class children appeared to have more undesirable eating habits.

Introduction

Food intake is a result of the dynamic interaction of many aspects not only of an individual's psychology, physiology and pathology but also the prevailing technical, sociopolitical and economic climate. Accordingly food habits have undoubtedly been changing both recently (Angel & Hurdle, 1978) and over a much longer time (Drummond & Wilbraham, 1958). A knowledge of food intake, and particularly its changes, is important to both epidemiological studies of nutritional status and health education. It is remarkable how few dietary surveys, particularly age-specific surveys in children, have been carried out in the UK. The only continuous survey (the national food survey) has several severe limitations, not the least being that it is not an estimate of intake, only acquisition (Derry & Buss, 1984).

The aim of this report of the sources of energy, protein, fat and fibre in the diet of 11- to 13-year-old children, is primarily to contribute to the development of health education programmes for this target population, based on current recommendations (DHSS, 1978, 1979, 1984; NACNE, 1983).

Methods

Between September 1979 and July 1981, 405 children initially aged 11-12 years recorded all dietary intake for three consecutive days on five separate occasions. The children were from seven schools in south Northumberland.

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Each child was given a specially designed diary in which to record a description of all foods and drinks taken, the time of consumption and an estimate of the amount consumed in household measures. They were then individually interviewed on the fourth day in order to verify and enlarge upon the information provided. The child's description was used, with a selection of calibrated visual aids, to arrive at a quantitative estimate of intake. Computerized food tables (Paul & Southgate, 1978), supplemented and modified as necessary, were then used to calculate the intake of a variety of nutrients (Hackett, Rugg-Gunn & Appleton, 1983). The fibre content of chips (code 645) is not given in these food tables. These data were kindly supplied by Dr D.A.T. Southgate, and included in the current analysis as a mean fibre content of 3.0 g/100 g.

To simplify the presentation of results, the foods consumed have been aggregated into a number of arbitrary groups, the components of which were largely determined by the groups given in *The Composition of Foods*, as follows: (1) the meat group included any pastry consumed as part of the food item, e.g. pork pie, while (2) the egg group did not include those eggs used in baking; (3) 'confectionery' was taken to include sweets, chocolate bars, popcorn, iced-lollies, ice-cream and full-coated chocolate biscuits; (4) 'soft drinks' included carbonated and still drinks but not tea, coffee, milk shakes or alcoholic drinks; and (5) 'sweet puddings' included tinned and stewed fruits and yoghurts. Table sugar refers to sucrose used as such in the home. More detail has been given on several of these groups elsewhere (Hackett *et al.*, 1984*a*). The groups chosen vary slightly between the different analyses in order to provide information on foods of particular relevance to each nutrient studied. For example the intake of baked beans is relevant to protein and fibre intake but not to energy or fat intake. Similarly the spreading fats are relevant to energy and fat intake but not to fibre or protein intake.

The weights of the individual food items consumed were used to calculate the amount of energy, protein, fat or fibre derived from that source. These were then combined into the groups already outlined and expressed as a proportion of the total intake. The information presented in this paper is given as the average daily intake per child. Similar data concerning the sources of sugars and calcium intake have already been published (Hackett *et al.*, 1984*a*,*b*).

The occupation of each child's father was coded as social class (Registrar General, 1970). In order to increase the size of the groups analysed, classes I + II were grouped together and labelled 'high' (professional occupations), III (alone) was labelled 'middle' (skilled workers), and IV + V, 'low' (manual workers).

Results

Four hundred and five children completed all aspects of the study: 52 per cent of those invited to participate. Nine boys and 21 girls were not classified into social classes I to V and have been excluded from further analyses. Although all data for energy, protein, fat and fibre were analysed by sex and

social class, Tables 1 to 4 present data for all children and by sex only. Important inter-social class differences are given in the text.

The boys' energy intake was greater than that of the girls' ($P < 0.05$) but the contribution of protein (11 per cent) and fat (40 per cent) to the energy intake in each sex was similar and neither did this composition change over the two year study period (Hackett *et al.*, 1984*c*). There was, however, a very wide range of mean intakes as indicated by large coefficients of variation. There were no significant differences between the major nutrient intakes of the social classes within each sex (Hackett *et al.*, 1984*c*).

The contributions of various arbitrary food groups to the energy intake are shown in Table 1. Potatoes contributed the largest single component of

Table 1. *The energy intake (mean ± s.d.) and the percentage contribution of groups of foods to the energy intake of 375 adolescents, by sex.*

Sex	*Boys* (*n* = 184)	*Girls* (*n* = 191)	*All* (*n* = 375)
Energy intake (MJ)	9.5 ± 1.47	8.5 ± 1.43	8.9 ± 1.51
Food group	%	%	%
Meat	13.7	12.3	13.1
Bread	10.3	10.0	10.2
Biscuits & cakes	8.5	9.5	9.0
Chips	9.2	8.7	9.0
Confectionery	7.6	8.6	8.2
Milk	8.5	7.0	7.8
Sweet puddings	5.4	5.8	5.6
Butter & Margarine	5.0	4.6	4.8
Crisps	3.4	5.3	4.4
Table sugar	3.6	3.1	3.4
Breakfast cereals	3.4	2.8	3.1
Other potatoes	2.8	2.9	2.9
Soft drinks	2.4	3.1	2.8
Cheese	0.8	1.5	1.2
Other	15.4	14.8	14.5

the energy intake (16.3 per cent) but this was due to some extent to the form in which they were eaten, as chips and crisps both have a high fat content. The intake of chips was related to social class: the weights eaten for each class, boys and girls combined, were 'high' 54 g, 'middle' 78 g and 'low' 90 g. The girls consumed proportionately more energy as crisps than the boys. Although bread made an important contribution to energy intake, so did biscuits and cakes. Most of the bread consumed was white; the intake of all 'brown' breads was very low and mainly consumed by the children of high social class ('high' 13.5 g, 'middle' 7.3 g, 'low' 4.0 g). Flour-based products (including some sweet puddings) therefore contributed considerably to energy intake but the preferences were towards sweet and/or fatty foods. Confectionery, table sugar and soft drinks also made significant contributions to the energy intake of all groups of children, with the contributions of confectionery and table sugar being slightly higher in social classes IV + V ('high 7.5 per cent, 2.7 per cent, 'middle' 7.8 per cent, 3.5 per cent, 'low' 8.9

per cent, 3.8 per cent respectively). The remaining energy not accounted for (probably mostly due to fruit and vegetables) contributed 3-4 per cent less to the diets of the lower social class children.

'Meat', milk, bread and potatoes contributed 61 per cent of the protein intake (Table 2). Cheese was a more important source of protein in the girls, particularly those in social classes I + II (girls: 'high' 6.0 per cent, 'middle' 3.4 per cent, 'low' 2.0 per cent; boys: 'high' 2.7 per cent, 'middle' 1.4 per cent and 'low' 1.6 per cent). The contribution of breakfast cereals was higher in social classes I + II in both boys and girls ('high' 4.2 per cent, 'middle' 3.1 per cent and 'low' 2.4 per cent). Individual vegetables apart from potatoes, baked beans and peas made only a minor contribution to protein intake. The

Table 2. *The protein intake (mean ± s.d.)and the percentage contribution of groups of foods to the protein intake of 375 adolescents, by sex.*

Sex	*Boys (n = 184)*	*Girls (n = 191)*	*All (n = 375)*
Protein intake (g)	64 ± 10.5	56 ± 10.3	60 ± 11.2
Food group	%	%	%
Meat	28.1	26.8	27.5
Milk	15.1	12.9	14.0
Bread	11.7	11.9	11.8
Potatoes (all)	7.8	8.2	8.0
Sweet puddings	4.4	4.7	4.6
Biscuits & cakes	4.4	4.5	4.5
Fish	3.9	3.8	3.9
Eggs	3.5	4.0	3.8
Breakfast cereals	3.5	3.1	3.3
Cheese	1.9	3.8	2.9
Confectionery	2.1	2.4	2.3
Baked Beans	2.2	1.9	2.1
Peas	1.2	1.2	1.2
Nuts	0.5	0.6	0.6
Other	13.2	10.2	9.5

contributions were highest in social classes IV + V for potatoes ('high' 6.5 per cent, 'middle' 8.3 per cent, 'low' 9.2 per cent), baked beans ('high' 1.3 per cent, 'middle' 2.1 per cent, 'low' 2.7 per cent), and peas ('high' 0.9 per cent, 'middle' 1.3 per cent and 'low' 1.4 per cent).

As the type of fat consumed was thought unlikely to influence the development of dental caries (the main purpose of the study) no attempt was made to collect the data in such a way as to enable the calculation of the fatty acid composition of the diet. Total fat intake only is available. Table 3 shows the origin of the fat consumed: 'meat', spreading fats and milk made the major contributions, but potatoes (as chips and crisps), confectionery (chocolate and ice-cream), biscuits and cakes and sweet puddings were also important. Only a minor contribution from cheese was found. Pastry (in various foods) was found to contribute on average 6.2 per cent of the total fat intake.

The figures for fibre intake differ from those in a previous publication

Table 3. *The fat intake (mean ± s.d.) and the percentage contribution of groups of foods to the fat intake of 375 adolescents, by sex.*

Sex	*Boys (n = 184)*	*Girls (n = 191)*	*All (n = 375)*
Fat intake (g)	101 ± 18.4	92 ± 16.8	97 ± 18.0
Food group	%	%	%
Meat	21.1	18.5	19.9
Butter & Margarine	14.6	14.6	14.6
Milk	10.8	9.1	10.1
Biscuits & Cakes	8.4	9.4	8.9
Chips	8.9	8.3	8.6
Confectionery	6.1	6.4	6.3
Crisps	5.1	7.2	6.2
Sweet puddings	4.1	4.1	4.1
Eggs	2.8	3.0	2.9
Bread	2.3	2.2	2.3
Cheese	1.6	2.7	2.2
Nuts	0.8	1.2	1.0
Fish	1.0	0.8	1.0
Breakfast cereals	0.8	0.6	0.7
Cream	0.3	0.3	0.3
Other	11.3	11.6	10.9

(Hackett *et al.*, 1984*c*) since the contribution from chips is now included (Table 4). Chips were the major source of fibre overall partly due to the large amount consumed by the children in social classes IV + V (weights for each social class have already been given). Several groups of foods supplied substantial amounts of fibre: besides chips, the contributions of crisps and baked beans were particularly striking. The contribution of baked beans was especially marked in social classes IV + V ('high' 7.8 per cent, 'middle' 11.2 per cent and 'low' 13.8 per cent). Breakfast cereals were also important with

Table 4.*The fibre intake (mean ± s.d.) and the percentage contribution of groups of foods to the fibre intake of 375 adolescents, by sex.*

Sex	*Boys (n = 184)*	*Girls (n = 191)*	*All (n = 375)*
Fibre intake (g)	17.0 ± 3.8	15.6 ± 3.6	16.3 ± 3.8
Food group	%	%	%
Chips	14.4	13.6	14.0
White bread	13.3	11.8	12.6
Crisps	9.8	14.2	12.0
Baked beans	12.5	10.3	11.4
Breakfast cereals	12.2	9.2	10.7
Vegetables (not potatoes)	10.9	10.3	10.6
Fruit	5.1	7.6	6.3
Biscuits & Cakes	5.3	5.7	5.5
Other potatoes	4.2	3.7	3.9
'Brown' breads	2.4	3.6	3.0
Sweet puddings	2.9	2.9	2.9
Nuts	0.6	0.9	0.8
Other foods	6.4	6.2	6.3

respect to fibre and there was a social class gradient ('high' 13.4 per cent, 'middle' 10.7 per cent and 'low' 8.7 per cent), but fruit (excluding tinned which were included with 'puddings'); made a relatively minor contribution. The social class gradient of the contribution of 'brown' breads was also impressive ('high' 5.6 per cent, 'middle' 2.7 per cent and 'low' 1.5 per cent). Chips and crisps together supplied about a quarter of these children's fibre intake, with higher proportions in social class IV + V children ('high' 21.1 per cent, 'middle' 26.7 per cent and 'low' 28.3 per cent). Vegetables were found to account for approximately two-thirds and cereals one-third of the total fibre intake.

Although alcohol made an insignificant contribution to the mean energy intake of these 11-14 year old children (0.04 per cent), some alcoholic drinks were recorded during each of the five surveys. They were mostly associated with Christmas while other festivities accounted for most of the remainder. A total of 75 children reported an alcoholic drink at least once during the 15 days surveyed, 10 reported having more than one during any one 3-day survey — and 23 reported alcohol intake at more than one survey. The drinks reported included (in order of frequency): wine, beer and cider, fortified wine, and spirits. Some liqueurs were consumed in chocolates.

Discussion

The longitudinal nature of the study, five dietary surveys plus three dental examinations (with X-rays) within the 2-year period, may have contributed to the low completion rate. The sample of children who completed the study cannot therefore be considered to be random, nevertheless all social classes and both sexes remained well represented. The design and conduct of this survey together with estimates of validity and reliability have been reported previously (Hackett *et al.*, 1983, 1984*c*, 1985).

There are two other British surveys of the intake of adolescents which provide comparable information. Widdowson's study of the diet of pre-war children (Widdowson, 1947) contained much information concerning the weight of foods consumed and their contribution to energy intake. In her study, potatoes contributed less than 5 per cent of the energy intake (all figures are the average for the seventy 11-, 12- and 13-year-old subjects unless otherwise stated), but this probably does not include any fat absorbed during cooking. However, only approximately 21 per cent of the children of all ages surveyed, recorded eating chips during the 7-day survey (99 per cent did so in the present 15-day survey) and only 2 per cent recorded eating crisps during the 7-day survey (98 per cent in the present 15-day survey). This may be a consequence of changes in many aspects of society: food technology, take-away food, school meals, domestic equipment and the increased tendency to eat 'snacks' rather than meals. Some other indications of changes in the dietary habits of children include the observation that 10 per cent of Widdowson's sample ate junket, 6 per cent milk jelly and 3 per cent barley pudding, none of which were recorded in this study 40 years later. Furthermore, only five of Widdowson's 916 subjects ate margarine

compared with 71 per cent of our sample. Bread (all types) supplied a slightly greater proportion of the energy in the pre-war study (15-16 per cent) compared with 10 per cent in this study (Table 1). Brown breads, however, comprised approximately 20 per cent of the total bread intake of the children in Widdowson's study compared to 16 per cent for the 'high' social class children, 9 per cent for the 'middle' and 5 per cent for those of lower social class in the present study. Thus the consumption by children of bread generally, and 'brown' breads in particular, has fallen. Calculations from Widdowson's data suggest that milk contributed about 18 per cent of her subjects' total protein and 15 per cent of their fat intake. This may indicate the fall in the importance of milk in the diet during adolescence although the pre-war study was mainly of 'middle class' children. Widdowson reported that biscuits, confectionery and particularly cakes were popular.

Only one child (1.4 per cent) consumed any alcohol (cider) during the 7-day survey. This compares to our finding measured over 15 days, that 75 children (18.5 per cent) took a variety of alcoholic drinks including fortified wines and spirits. Bull (1985) found that 39 per cent of 18- to 21-year-old adolescents reported an alcoholic drink 1-3 times per week. These findings indicate a possible area for concern and show that the experience of a variety of alcoholic drinks can begin at a very young age. Truswell & Darnton-Hill (1981) commented on the lack of information concerning the 'drinking' habits of adolescents (girls aged 13-17 years, boys 15-21 years). They concluded that alcohol abuse is the most serious disorder of food habits at this age and is related to the leading cause of death (accidental) in this age group. These comments together with our data suggest that education as to the effects of alcohol and its sensible use is important from an early age.

Durnin *et al.* (1974) gave the food sources of energy and protein of 14-year-old Glaswegian children in 1964 and 1971: a comparison of these data with our 1979-1981 study reveals several similarities and some possible trends. The downward trends in the percentage contribution to energy intake for 1964, 1971 and 1980 respectively for milk (14, 10, 8), bread (13, 11, 10), and cakes and biscuits (13, 10, 9) are consistent with the trend in national figures (MAFF, 1985) but are not conclusive evidence for this particular age group. The contribution of potatoes appears to have risen, 9, 11 and 16 per cent in 1964, 1971, and 1980 respectively. The components of the groups of foods in Durnin's study may be different to those in the present study and there may be regional differences in intake.

Durnin *et al.* (1974) reported no differences between the sexes and social groups. In the present study, children in social classes IV + V had more undesirable eating habits than the other social classes, for example, the highest consumption of chips and the lowest consumption of 'brown' breads. There was also some evidence of increased use of table sugar and confectionery and a lower intake of fruit and vegetables other than potatoes in these social class IV + V children. The food habits of the boys differed in some respects from those of the girls: notably the boys had a smaller contribution to energy from crisps. Bull (1985) also reports differences between sexes and social classes in the intake of some foods. However, the

analyses reported by Bull are different from those in this paper, preventing many direct comparisons.

A reduction in the intake of fat and sugars, and an increase in fibre is desirable (DHSS, 1978; NACNE, 1983). Black, Ravenscroft & Sims (1984) assessed the appropriateness of the NACNE (1983) dietary goals by comparison with the dietary intake of a group of dietitians. Their report indicated several difficulties involved in giving dietary advice. One problem was associated with making recommendations for a single dietary component since the diet as a whole should be considered and, second, the problem of extrapolating recommendations for the national diet to sub-groups of the population and especially to individuals. They concluded that the actual amounts of nutrients recommended are of little importance to practical health education, which can only be formulated in terms of foods. As Blaxter (1980) succinctly wrote: 'While some nutritionists ingest nutrients, most ordinary people eat food ....' The data in this paper indicate those food groups which deserve the attention of those who formulate health education. In addition, some foods considered nutritionally undesirable on account of their composition, were found to be relative minor components of the diets of these adolescents.

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Archs oral Biol. Vol. 29, No. 12, pp. 983–992, 1984
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0003-9969/84 $3.00 + 0.00
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RELATIONSHIP BETWEEN DIETARY HABITS AND CARIES INCREMENT ASSESSED OVER TWO YEARS IN 405 ENGLISH ADOLESCENT SCHOOL CHILDREN

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Summary—The study was conducted in seven schools on children initially aged 11.5 years. They recorded their diet on five occasions, each of three days, and received an annual dental examination, including radiography. Caries increments were low, mostly (58 per cent) in fissure surfaces. Correlations between caries increment and dietary factors were low due to the low caries increments observed and the large error associated with dietary data where analyses attempt to discriminate between individuals. The highest correlation was between caries increment and weight of daily intake of sugars ($+0.143$, $p < 0.01$). Multivariate analyses revealed that this relationship could not be explained by differences in sex, social class, tooth-brushing habits or level of plaque as measured by gingival inflammation. Weight of sugar intake appeared to be more strongly correlated to caries than frequency of intake; concentration of sugars in foods was positively related, and sugars in snacks were more strongly related to caries than total dietary sugars. The 31 children who consumed most sugar (> 163 g/day) developed 5.0 DMFS during the 2 years, 0.9 DMFS per year more than the 31 children (3.2 DMFS during 2 years) who had the lowest sugar intake (< 78 g/day).

INTRODUCTION

The evidence relating dietary sugars to dental caries is overwhelming (Newbrun, 1983; Rugg-Gunn, 1983). Although the evidence comes from many sources including animal experiments, plaque pH and *in vitro* laboratory experiments, it is important that surveys of human subjects are conducted to investigate which dietary habits are the most harmful and which are the most favourable to dental health. Interventional human studies, for example the Vipeholm (Gustafsson *et al.*, 1954) and Turku (Scheinin and Mäkinen, 1975) studies, are difficult to carry out as they are extremely expensive and it is not easy to maintain groups of people on specific diets for many years. Thus, much of the evidence in man has come from observational studies, all except one of which have been cross-sectional in design. Whereas a cross-sectional design may be satisfactory in young children, as age increases it becomes less satisfactory to compare life-time caries experience with a measure of diet which might refer only to the previous few days (Rugg-Gunn, 1981). It was therefore considered important to conduct a study where dietary habits and the development of dental caries were measured over the same period of time. Although this approach has been suggested by some authors (Sullivan and Goldsworthy, 1958; Koch and Martinsson, 1971; Bagramian *et al.*, 1974), it has been attempted only in a study by Clancy *et al.* (1977) in which the dietary habits of American children were assessed at one interview and correlated with the development of caries over the previous one year.

Methods of analysing data from observational studies of diet and dental caries have varied. The dietary habits of subjects with high and low caries experience have been compared (Duany, Zinner and Jablon, 1972; Martinsson, 1972; Kleemola-Kujala and Rasanen, 1979; Shaw and Murray, 1980; Shklair, 1981), whereas in others comparisons have been made of the caries experience of subjects with a high or low consumption of sugar or confectionery (Mansbridge, 1960; McHugh, McEwen and Hitchen, 1964; Bagramian *et al.*, 1974); a third way has involved regression analysis to correlate caries experience with defined dietary variables, but few studies have subjected these data to multiple regression analyses to rank dietary variables in the order in which they correlate with caries development (Richardson, Boyd and Conroy, 1977) or to take into account possible confounding factors (Granath *et al.*, 1978).

To avoid these shortcomings, we planned an observational incremental study where the diet of the subjects and their development of dental caries would be assessed over the same period. The following methodological decisions were made: (1) Adolescents were chosen because caries increment is highest and application of the findings of the study would have the greatest impact on the health of the community. (2) The study had to be sufficiently long to ensure adequate caries development but sufficiently short to ensure that each individual's dietary habits changed as little as possible; two years was the best compromise; a two-year caries increment was previously large enough (Rugg-Gunn, Holloway and Davies, 1973). (3) Factors other than diet could influence the incidence of caries; the study was therefore conducted in an area with a water supply low in fluoride (< 0.1 mg F/l) and where the use of fluoride tablets and mouth-rinses was negligible (A. D. French, 1978, personal communication).

SUBJECTS AND METHODS

The subjects

The subjects were children in their final two years at seven of the eight middle schools in the Ashington/Newbiggin/Morpeth area of south Northumberland. A letter was sent to parents informing them of the purpose of the study; only children whose parents consented were included: no reward was offered for participation. The eighth middle school participated in a subsidiary investigation to test the hypothesis that the children's knowledge of the dental aspects of the main study influenced their recording of their dietary habits [the so-called Hawthorne effect; Liu *et al.* (1978)]. The study was conducted between autumn 1979 and autumn 1981.

Data collection

The measurement of dietary intake was based upon a dietary diary in which the subjects recorded everything eaten or drunk during three consecutive days. Each child completed two three-day diaries between September 1979 and July 1980 and three three-day diaries between August 1980 and July 1981, thus recording a total of 15-days dietary intake over the two-year study period. The days on which the diary was to be completed were specified in order to ensure that all days of the week were represented during the two years for each child. The children were thoroughly instructed on how to complete the diaries and were individually interviewed in school on the day after completion of the diary in order to (1) clarify any uncertainties in the diary, (2) quantify the amount of food eaten (with the aid of food models, graduated cups etc.), (3) encourage the child to complete the study. Each diary contained (1) the food items eaten, (2) the time each item was eaten, (3) the quantity eaten (converted at interview to g weight), (4) whether it was eaten alone or in combination, (5) whether it was eaten as part of a meal or a snack. Bedtime was recorded for each day (Rugg-Gunn *et al.*, 1984). All dietary data were collected by one dietitian (Hackett, Rugg-Gunn and Appleton, 1983).

Each subject was dentally examined by one examiner in a purpose-built examining van in October 1979, 1980 and 1981. The caries-examining system was based on a visual system (Rugg-Gunn *et al.*, 1973) except that only one pre-cavitation grade (C1) and one cavitation grade (C3) were recorded. Bite-wing radiographs were taken by one radiographer and read by one examiner using 1.5 times magnification. The radiographic grading X1 (enamel only) was taken to be equal to the C1 clinical grade and the X2 radiographic grade (at the enamel–dentine junction) equivalent to the C3 clinical grade (Rugg-Gunn, 1972a). A bilateral recording system was used in which 71 per cent of sites were examined. The following were included:

Fissure sites. Occlusal surfaces of first and second premolars and first and second molars; palatal surfaces of maxillary first and second molars; buccal surfaces of mandibular first and second molars.

Approximal sites. Mesial and distal surfaces of first and second incisors, second premolars and first molars; mesial surfaces of canines and second molars; distal surfaces of first premolars.

Free smooth surfaces. Buccal surfaces of first and second maxillary incisors, first and second mandibular premolars; buccal surfaces of canines, and first and second molars; lingual surfaces of first and second mandibular molars; palatal surfaces of first and second maxillary molars.

At each annual examination the level of gingival inflammation was assessed using the G.I. criteria (Löe and Silness, 1963) on two sites per tooth (labial or linqual) on the following 6 teeth

		4	2	6	
6	2			4	.

The G.I. index was the mean score for the examined sites, and used as a measure of the average level of plaque accumulation for each child. Independent of the main survey, the tooth-brushing habits of these and other children in their schools were assessed over a three-day period by questionnaire on one occasion during the second year of the study by Health Authority personnel. Almost all children said they used a fluoride-containing toothpaste.

Father's occupation was obtained from school records or from parents and categorized into social class (Registrar General, 1980).

Data analysis

All the data were entered into computer store. With the aid of the computerized version of McCance and Widdowson's Food Tables (Paul and Southgate, 1978) the dietary data were converted into dietary variables for each of the 15 days recorded. Some updating of food tables was required, for example to include newly-introduced foods. The only sugar specified by name in the food tables was lactose, and sugars refers to the sum of all dietary sugars (almost entirely sucrose, lactose, glucose and fructose). The order in which foods were entered in the diary was assumed to be the order in which they were eaten.

The dietary variables were: energy intake, weight of macro-nutrients and micro-nutrients; the weight and frequency of eating of various categories of foods (e.g. confectionery, biscuits and cakes); the frequency of eating the same categories of food last in an intake; the weight and frequency of eating either foods or dietary items categorized according to their sugars content [(i) < 1.0 per cent sugars, (ii) 1.1–10.0 per cent sugars, (iii) 10.1–60.0 per cent sugars, (iv) >60.0 per cent sugars], and the length of time between an intake of food or dietary item (categorized according to its sugars content) and bedtime. A dietary item was defined as one which had a discrete McCance and Widdowson food code, e.g. milk or table sugar or an infusion of tea; a food was one or more dietary items eaten together, e.g. a cup of tea (containing milk and sugar and an infusion of tea). The concentration of sugars in dietary items was obtained directly from food tables; the concentration of sugars in foods was calculated from the weight of each item comprising that food and the concentration of sugars in those items. In calculating the frequency of dietary intake, foods or items eaten within 30 min of the start of a meal were deemed to be part of that intake; foods or items eaten within 15 min of a snack were classed as part of that intake, otherwise they were classed as separate dietary intakes.

Before undertaking the parametric statistical anal-

yses, non-normal data were transformed [to log (x), log ($x + 1$) or log ($x + 2$)] as required. The reliability of the measurement of total daily sugars intake was found to be 0.78 (Hackett *et al.*, 1983). The reliability of the measurement of dental caries was not assessed; it had been found previously to be 0.85 for similar data [two-year decayed, missing, filled surface index (DMFS) increment at the C3 level for 162 11.5–13.5-year-old children (Rugg-Gunn, 1972b)].

RESULTS

Four hundred and sixty-six children (59 per cent of those invited) volunteered to take part in the study. Fifty-four children left the area or were absent from part of the study; two children asked to leave the study and the diaries of five children were judged to be unreliable and were excluded from the analyses. Subsequent analyses were limited to the 405 children (193 boys, 212 girls) who completed all five three-day diaries and were dentally examined at the beginning and end of the two-year period. Their mean age was 11 years 7 months in October 1979. The percentage of children in each social-class group were: I + II 21 per cent, III 43 per cent and IV + V 29 per cent, with 7 per cent miscellaneous (social classes VI to VIII). Only one child was non-caucasoid.

Dietary results

The mean daily energy intake for all subjects in all five surveys was 8.9 MJ: 11 per cent of this was supplied by protein, 40 per cent by fat, and 48 per cent by carbohydrate. On average, 118 g (±29.4) of sugars were consumed per day, which supplied 21 per cent of the average daily energy intake. Further information on the subjects' nutrient intake was provided by Hackett *et al.* (1984a) and on sugar intake by Hackett *et al.* (1984b).

The weight and frequency of eating some categories of foods are given in Table 1. On average, each child consumed 57 g of confectionery (sweets + chocolates) per day; the frequency of intake averaged 1.8 intakes per day. By weight, 24 per cent (412 g) of foods and drinks consumed contained <1.0 per cent sugars, 57 per cent (977 g) contained between 1.1 and 10.0 per cent sugars, 18 per cent (302 g) contained between 10.1 and 60.0 per cent sugars, and 1 per cent (22 g) contained >60.0 per cent sugars. On average, there were 6.8 intakes of food or drink per day. The majority (6.0) of these intakes contained at least one food of >1 per cent sugars; 54 per cent (3.7 intakes) of the total number of intakes per day contained at least one food of >10 per cent sugars.

Dental results

At the beginning of the investigation (baseline), the mean (±SD) caries experience for the 405 children was 3.3 (±2.3) decayed missing filled teeth (DMFT) and 5.0 (±3.9) DMFS (C3 grade). The mean (±SD) two-year caries increment (C3 grade) was 2.2 (±2.1) DMFT and 3.6 (±3.6) DMFS; the positively-skewed frequency distribution of the DMFS increment is given in Fig. 1. Only 29 children (7 per cent) had a two-year DMFS increment of 10 or more. Fifty-seven per cent of this caries increment occurred in the fissure surfaces and 33 per cent in the posterior approximal surfaces (Table 2). The mean DMFS increment at the C1 grade was 9.0 (95 per cent range −2 to 25). The mean gingival index (G.I.) score (mean of baseline and final score for each child) was 0.80 (95 per cent range 0.21–1.55). Due to non-compliance, data on brushing frequency were available only for 219 (54 per cent) of the subjects; the mean number of times that they brushed their teeth over the three-day period was 5.6 times (95 per cent range 3–9).

Correlations between diet and caries

The correlations between weight of daily sugars intake and two-year caries increments are given in Table 2. There was a low statistically-significant positive correlation between DMFS increment and weight of sugars intake; the fissure surfaces were the most sensitive to dietary differences. The size of the correlation of sugars intake with different surface

Table 1. Means and 95 per cent ranges for weight and frequency of intake of some dietary variables obtained from the mean daily intake for 405 children (mean of 15 days per child)

	Weight (g)		Frequency (per day)	
Dietary variable	mean	95 per cent range	mean	95 per cent range
Sweets	22	2–61	1.0	0.1–2.6
Chocolate	35	6–80	0.9	0.1–2.1
Biscuits + cakes	45	8–107	1.0	0.2–2.3
Sweet puddings	75	8–167	0.5	0.1–1.2
Tea (unsugared)	64	0–452	0.3	0.0–2.1
Tea (sugared)	151	0–690	0.7	0.0–2.9
Other hot drinks (sugared)	110	0–580	0.6	0.0–2.4
Soft drinks (sugared)	60	0–189	0.7	0.0–2.1
Milk	250	52–595	2.3	0.5–4.0
Cheese	6	0–24	0.2	0.0–0.7
Apples (raw)	25	0–82	0.3	0.0–0.9
Other fruits + vegetables (raw)	30	0–96	0.5	0.0–1.3
All food + drinks	1713	1201–2336	6.8	4.3–11.7
Foods > 1.0 per cent sugars	1301	746–1950	6.0	3.9–10.0
Foods > 10.0 per cent sugars	324	138–618	3.7	1.7–6.4
Foods > 60.0 per cent sugars	22	2–57	1.0	0.1–2.7

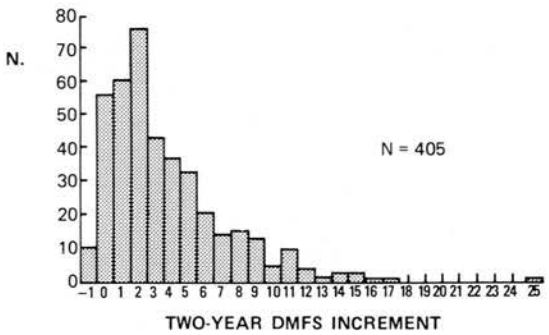

Fig. 1. Frequency distribution of the two-year DMFS increments (C3 level: excluding pre-cavitation lesions) for 405 subjects, initially aged 11.5 years.

types corresponded to the size of the increments in those surface types (Table 2). Generally, the correlations between weight of sugars intake and caries increment scores at the C1 diagnostic level were lower than at the C3 level, except for anterior approximal surfaces where the correlations were slightly, but not significantly, higher. The correlation between the baseline DMFS (at C3 grade) and the weight of total sugars intake obtained at the first of the five dietary surveys only, was +0.005.

The bivariate correlation coefficients which were statistically significant between two-year fissure caries increment and dietary variables are given in Table 3. The highest correlation (+0.143) was for weight of total daily sugars. A negative correlation was observed for the length of time between the last food intake, which contained more than 10 per cent sugars, and bedtime. When the data were subjected to stepwise regression analysis and the weight of total sugars was included in the analyses, no other dietary variable increased the multiple R by a statistically-significant amount. The influence of possible confounding factors on the relationship between fissure caries increment and weight of total sugars, was investigated. The correlations between these two variables were similar in the boys (+0.136, $p = 0.06$) and girls (+0.140, $p = 0.04$). Correlations were higher (although not significantly) for the 118 children in social class IV + V (+0.139, $p = 0.13$) than for the 85 in social class I + II (+0.090, $p = 0.41$) or the 172 in social class III (+0.095, $p = 0.21$) although all were positive. The correlation between fissure-caries increment and gingival index (mean of baseline and final examination scores) was +0.128 ($p < 0.01$). Differences in the amount of plaque accumulated (as indicated by the G.I.) could not explain the relationship between caries and sugars intake, as the partial correlation between fissure-caries increment and total daily sugars controlling for gingival index was +0.146, similar to the bivariate correlation of +0.143 when the data were not controlled for gingival index. Likewise, when the data were controlled for the variable tooth-brushing frequency, the correlations between fissure-caries increment and total daily sugars was +0.165 ($p = 0.02$, $n = 219$), higher

Table 2. Mean (and 95 per cent range) two-year caries increment for various indices at the C3 grade, and the percentage of the total caries increment which occurred in each type of surface (fissure, approximal and free smooth surface), together with the correlations between the caries increments and the weight of daily sugars intake for 405 children

Caries index	Two-year increment		Percentage of total carious surfaces	Correlation
	mean	95 per cent range		
DMFT	2.20	0–7		+0.077 (NS)
DMFS	3.63	0–12		+0.105 ($p < 0.05$)
DFS fissure)	2.10	−1–7	57	+0.143 ($p < 0.01$)
DFS (posterior approximal)	1.22	0–6	33	+0.045 (NS)
DFS (anterior approximal)	0.13	0–2	3	+0.004 (NS)
DFS (free smooth surface)	0.24	0–2	7	−0.010 (NS)

Table 3. Ranking of dietary variables according to their bivariate correlation with two-year fissure caries increment

+0.143*	Total sugars (weight)
+0.129*	Total sugars, excluding lactose (weight)
+0.118	Items > 10 per cent sugars (weight)
+0.116	Sweets + chocolate + biscuits + cake + puddings (weight)
+0.115	Items > 60 per cent sugars (weight)
+0.112	Foods > 10 per cent sugars (weight)
+0.111	Sweets (weight)
+0.108	Sweets + chocolate (weight)
+0.108	Hot sugared drinks (excluding tea) (frequency)
+0.103	Lactose (weight)
+0.102	Milk (weight)
+0.101	Items > 1 per cent sugars (weight)
+0.099	Items > 10 per cent sugars (frequency)
+0.099	Sweets + chocolate + biscuits + cake + puddings (frequency)
+0.098	Foods > 1 per cent sugars (weight)
−0.101	Time between last food > 10 per cent sugars and bed

Only statistically significant correlations are shown (*$p < 0.01$, the remainder $p < 0.05$).

Table 4. Bivariate correlations* between two-year caries increment and some dietary variables† when measured (a) total amount consumed per day (b) amount consumed as snacks‡ only

	DMFS (all surface-types)		DFS (fissure surfaces only)	
	All intakes	Snacks	All intakes	Snacks
Total sugars	0.105§	0.132‖	0.143‖	0.146‖
Sweets and chocolate	0.085	0.124§	0.108§	0.111§
Biscuits + cakes + puddings	0.024	0.106§	0.069	0.123§
Sweets + chocolate + biscuits + cake + puddings	0.069	0.142‖	0.116§	0.146§
All sugared drinks	0.033	0.016	0.059	0.044

*All positive. †All weights of foods consumed. ‡Defined as an intake having at least one of the following: (i) thought to make a minor contribution to the day's intake; (ii) eaten whilst engaged in some other activity; (iii) not eaten at recognized mealtimes or places. §$p < 0.05$. ‖$p < 0.01$.

than the corresponding bivariate correlation where tooth-brushing frequency was not taken into account.

The statistically-significant correlation (−0.101) between fissure-caries increment and the interval between eating a sugary food and bedtime (Table 3) fell to a non-significant value of −0.032 ($p = 0.52$) when the data were controlled for weight of sugars consumed per day.

There was statistically-significant correlation between weight of milk consumed and fissure-caries increment (+0.102). However, when these data were controlled for weight of sugars in the diet other than lactose, the correlation fell to +0.086 which, although no longer statistically significant, was still positive.

For most dietary variables, the correlation between fissure-caries increment and the weight of the dietary variable was higher than the correlation between fissure-caries increment and frequency of consumption of that same dietary variable (Table 3 and data not shown).

The correlations in Table 3 refer to total intakes which could have been as meals or snacks. When the dietary variables were calculated for snacks alone the correlations with caries increment were usually higher (Table 4). The correlations for caries increment with sugared drinks were lower than for caries increment and sweets, chocolate, biscuits, cakes and puddings (Table 4).

A relationship between fissure-caries increment and the concentration of sugars in dietary items was observed. Total weight of items consumed per day containing low concentrations (< 1 per cent) of sugars was weakly-negatively correlated to caries increment (−0.002). The size of the positive correlations increased as the concentration of sugars in the dietary items increased: for sugars concentration 1.1–10.0 per cent, $r = +0.057$; for sugars concentration 10.1–60.0, $r = +0.096$, and for sugars concentration of > 60.0 per cent, $r = +0.115$.

No tendency was observed for correlations between caries increment and frequency of sugars when last in an intake to be higher than correlations between caries increment and frequency of all sugars intake.

Caries increment in high and low sugars eaters

A comparison of the mean caries increment in the 31 children (7.5 per cent) eating the highest amount of sugars (> 163 g/day) with the 31 children (7.5 per cent) eating the lowest amount of sugars (< 78 g/day) revealed that more caries developed in the high sugars eaters (Table 5). This difference amounted to 1.78 DMFS over the two-year study period or approx. 0.9 tooth surfaces per child per year. Expressed in percentage terms, the low-sugar eaters developed 36 per cent less caries than the high-sugar eaters or, alternatively, the high-sugar eaters developed 56 per cent more caries than the low-sugar eaters (the denominators being different in the two calculations). The difference in DMFS increment was not statistically significant ($p = 0.07$); the difference in fissure surface caries increment of 1.13 surfaces over the two years was ($p = 0.03$). The greatest percentage difference (81 per cent) was in anterior approximal surfaces, but was not statistically significant.

Regression of DMFS increment on weight of total sugars indicated that there was an average increase of 1.28 DMFS over two years with each rise of 100 g sugars ($p < 0.01$, 95 per cent confidence range = 0.10–2.46 DMFS).

Table 5. The mean two-year caries increment of the 31 children with the highest and 31 children with the lowest mean weight of daily sugars intake, divided by the 7.5 and 92.5 percentiles

	Weight of daily sugars intake			
	High (> 163 g)	Low (< 78 g)	Difference	(per cent)
DMFS	4.97	3.19	1.78	(36)
DFS (fissure)	2.94	1.81	1.13*	(38)
DFS (posterior approximal)	1.52	1.26	0.26	(17)
DFS (anterior approximal)	0.16	0.03	0.13	(81)
DFS (free smooth surface)	0.42	0.26	0.16	(38)

*$p < 0.05$.

Table 6. Comparison of the mean daily dietary intake (g) of the 65 children who developed no caries during the two-year study with the 71 children who developed the highest caries increment (△DMFS ⩾ 7)

Dietary variable (g)	Zero-caries increment	High-caries increment	p (2-tail)
Sugars	113	122	0.09
Confectionery	53	62	0.05
Unsugared tea	88	38	0.06
Sugared tea	140	163	0.46
Other sugared hot drinks	75	115	0.05
Soft drinks	60	57	0.76
Milk	242	269	0.30
Cheese	7	5	0.07
Raw apples	23	28	0.27

Diet in caries-free and high-caries children

Sixty-five children developed no new carious lesions during the two-year study. Compared with 71 children with the highest two-year caries increment (⩾7 DMFS) (Table 6), their confectionery consumption was slightly higher ($p = 0.05$) and cheese consumption slightly lower ($p = 0.07$). The average consumption of unsugared tea was twice as great in the children with zero caries increment ($p = 0.06$), sugared tea consumption was virtually the same in the two groups but consumption of other sugared hot drinks was higher in the children with the high-caries increment ($p = 0.05$). The frequency of consumption of hot sugared-drinks (other than tea) was nearly twice as high (7 compared with 4 intakes per day) in the high caries-increment children ($p = 0.01$). Nineteen children were caries-free at the beginning, developed no carious lesions during the study and were thus caries-free at age 13 years 7 months. Although they constitute a small group, their diet was compared with the 23 children who had the highest caries experience (⩾ 20 DMFS) at age 13 years 7 months. The only statistically-significant difference in diet between the high-caries experience and the caries-free children was that the caries-free children had twice the cheese intake compared with the high caries children (8 g compared with 4 g per day, $p = 0.02$).

DISCUSSION

Because longitudinal observational studies of diet and dental disease are rare, the problems of data collection, design and analysis will be discussed.

The subjects

The acceptance rate (59 per cent) is low compared with previous studies. The most likely reasons are (1) the longitudinal design (most epidemiological studies of dental caries have been cross-sectional) and (2) the lack of perceived benefit by parent and child; in longitudinal clinical trials there is an opportunity to benefit from the preventive or therapeutic agent under test. The low acceptance rate was compensated by the low drop-out rate; only two children left the study. Although the 405 children (52 per cent of those asked) completing all aspects of the study were well distributed between the social classes, it is not known how they differed in caries status or dietary habits from the children who either did not volunteer or who did not complete all aspects of the study.

Diet-collection method

Measurement of voluntary food intake is notoriously difficult (Garrow, 1974) and is demanding on both subjects and investigators. Most dietary surveys of adolescents in the U.K. have involved weighing of food intake (Darke, Disselduff and Try, 1980), but this was impractical in our survey of over 400 children; our three-day diary system, repeated five times, was a reasonable alternative. The method is economical (Black, 1982) and reliable (Hackett *et al.*, 1983).

It was possible that the children would alter either their eating habits or their reporting because, for ethical reasons, they knew the purpose of the study. The subsidiary study at a separate school found no evidence that knowledge of the dental nature of the project altered the reported food intake (data not presented). Although the total weight of food intake appears to have been accurately recorded (Hackett *et al.*, 1984a), it is uncertain whether the intake of smaller items of the diet, and therefore the frequency of intake, was accurately recorded as no comparable British data exist on the frequency of intake. The mean number of food intakes per day was 6.8 (Table 1) which is considerably higher than the 3.9–4.3 intakes per day reported for American teenagers by Hampton *et al.* (1967); it is similar to the modal 6–8 intakes per day recorded by Frank, Berenson and Webber (1978) in American 10-year-old children.

Sugar and confectionery intake

The mean total-sugar consumption of 118 g per day included both natural and added sugars and provided, on average, 21 per cent of the energy intake in these young adolescents. As nutritional studies have seldom reported total sugar intake, these figures (118 g/day ≡ 43 kg/year) cannot be compared with recent data for British adolescents but they are marginally lower than the 46 kg/year per person sugars consumption estimated for the whole of the U.K. population in 1980 (M.A.F.F., 1983). Mansbridge (1960) reported that 12–14 -year-old Scottish children consumed about 8 oz of sweets and chocolate per week (≏32 g/day); McHugh *et al.* (1964) found that similarly-aged (13 years) Scottish children consumed a much higher amount, about 17.5 oz of sweets per week (≏70 g/day). These figures can be compared with the 22 g of sweets and 35 g chocolate per day in our study (Table 1).

Caries increment

The caries increment of 3.6 DMFS over two years was lower than the two-year increment of 5.9 DMFS recorded by the same examiner in similarly-aged Manchester school children 10 years previously (Rugg-Gunn *et al.*, 1973). The baseline DMFS was 8.8 in Manchester in 1968/69, and 5.0 in our study in 1979. Both studies were in low-fluoride areas, and this apparent decline in caries accords with the well-documented recent fall in caries prevalence in England (Anderson *et al.*, 1982). However, our study was planned in 1977, before that decline had become apparent, and there was no reason to predict that the caries increment would be smaller than that recorded in previous clinical trials. The low caries increment recorded cannot be explained by the use of partial recording, as this system, tested on data collected on similarly-aged children in Cheshire and Salford between 1975 and 1977, included 95–97 per cent of the baseline full-mouth DMFS and 95–96 per cent of the subsequent two-year DMFS (C3 grade) increment (T. G. H. Davies, M. A. Lennon and F. Teagle, 1980, personal communication). The range of two-year DMFS increments was only slightly smaller with partial recording than with full-mouth recording (0–38 to 0–35 in one study and 0–25 to 0–23 in the second study), indicating that it is unlikely to affect correlation analysis. To measure the maximum caries increment, it would have been preferable to have examined the children one year later (mean age 12.5–14.5 years). We rejected this because the children would have moved from Middle to High school between the first and second year, increasing the likelihood of a change in dietary habits.

Correlation analysis and study design

The highest correlation between the caries increment and a dietary variable (+0.143 for weight of sugars intake Table 3), although statistically highly significant, is low. Using multiple regression, no other dietary factor increased this correlation significantly. Of the non-dietary variables studied (sex, social class, gingival index and toothbrushing frequency) only gingival index increased the multiple R by a significant amount (to 0.193). Squaring the correlation provides an estimate of the amount of the variance in caries increment that is explained by the independent variables. Thus, total sugars explained only 2.0 per cent of the variance, while total sugars and G.I. explained only 3.7 per cent of the variance, leaving the remaining 96.3 per cent of the variance unexplained. Multiple regression is a powerful and theoretically useful method of analysis of epidemiological data and the present study was designed with this in mind. There would seem to be two main explanations of this low correlation. First, that dietary factors were unimportant in caries development in this group of people, compared with other factors which might influence caries such as: enamel composition, salivary, plaque and immunological properties, or exposure to prophylactic agents such as fluorides. This explanation is difficult to accept in view of the large amount of evidence from many sources which incriminates dietary sugars in caries aetiology. The second explanation is that a large proportion of the variance is due to method errors including large intra-subject variance which have masked a true relationship: increases in these sources of variance would decrease the correlation. Method errors can be discussed under four headings:

(i) The variability of the dietary factors and the low-caries increment. Subsequent simulation analyses (not presented here) indicate that the size and range of the caries increment influences the size of the correlations between the caries and dietary variables: the larger the caries increments the higher the correlations obtained. This possibility was foreseen, and the study was therefore conducted in a low-fluoride area (to obtain a large range of caries increments), and with children from a wide range of social classes (to increase inter-subject variation in dietary habits). Nevertheless, the small size of the correlations can partially be explained by the small size of caries increments. A further factor is that in dietary surveys, ranges of dietary intakes tend to be reduced by the so-called flat-slope syndrome, where there is a tendency for large intakes to be under-estimated and small intakes over-estimated (Maddern, Goodman and Gutherie, 1976). The important possibility that more extreme dietary habits or higher-caries increment might have been present in the 48 per cent of children who did not volunteer or complete the study has been commented upon.

(ii) Any reduction from unity in the validity and reliability of the variables studied would decrease the correlation coefficients obtained. The validity (Downer, 1975) and reliability (Rugg-Gunn, Downer and Ashley, 1976) of the caries-diagnostic system have previously been high; nevertheless the finding that correlations between dietary factors and caries increment when measured at the C1 grade were lower than at the C3 grade might be due to the lower reliability of caries data when pre-cavitation lesions are included. The validity of the dietary assessment was shown to be satisfactory for groups; the reliability of the measurements of sugars intake was 0.78. However, correcting the correlation between caries increment and diet for unreliability of the dietary and caries data resulted in only a small increase in the correlation coefficient (from 0.143 to 0.178).

(iii) For correlation analysis, the dietary variables had to be averaged down to one day's mean intake. Although calculations revealed that intra-subject variance was moderate, it is not known whether differences in dietary intake at different times had an unequal effect on caries aetiology. It might be argued that, as carious lesions develop slowly, high sugar intake earlier in the two-year period might be more damaging than a high sugar intake shortly before the final examination.

(iv) Any violation of the assumptions underlying parametric correlation analyses can result in an erroneous estimate of correlation. However, the robustness of correlation analysis was demonstrated in that coefficients calculated using transformed data were little different from those obtained using original data (+0.131 compared with +0.143 for fissure caries increment and weight of total sugars).

Since we planned our study Jacobs, Anderson and Blackburn (1979) reported on the value of correlation

analysis in studies of diet and serum cholesterol, concluding that, with commonly-used methods of dietary intake estimation, intra-individual variation is likely to be so great that the strength of the relationship between diet and serum cholesterol is likely to be seriously underestimated by such analysis techniques, in cross-sectional studies especially. It seems therefore that in our study, however attractive correlation analysis might be, comparison of groups of people (where the influence of intra-individual variation is reduced) is a more informative method of estimating the importance of diet in caries aetiology if no third variable is introduced. Although correlation analysis gives a poor guide to the importance of diet in caries aetiology, it is of value in determining whether confounding factors influence this relationship.

Inter-group comparisons

Substantial differences between the caries increments of the high and low sugars eaters were observed (Table 5). Regression analysis showed no indication of any plateau effect and, if a straight line relationship is assumed, a mean rise in two-year caries increment of 0.13 DMFS occurred with each 10 g increase in daily sugars consumption.

The diets of the children who developed either no caries or much caries during the study showed some interesting differences (Table 6). The consumption of unsugared and sugared tea and other hot drinks was considerably different although, because of large inter-subject variation in consumption, the differences were of borderline statistical significance. In contrast, there was virtually no difference in soft-drink consumption between groups and this agrees with the low correlations observed between soft-drink consumption and caries increment. The inference that the addition of table sugar to hot drinks may be more cariogenic than sugar in soft drinks agrees with plaque-pH studies in which sugared tea and coffee produced longer and deeper Stephan curves than the more sialogogic fruit cordial (Rugg-Gunn, Edgar and Jenkins, 1978). Differences are not great enough to conclude that the fluoride content of tea exerted a cariostatic effect, although the trend was in that direction. Likewise, the finding that cheese consumption (although small in absolute amounts) was statistically-significantly higher in the caries-free children than in the children with the highest caries experience at age 13.5 years, supports the conclusions of studies on plaque pH (Rugg-Gunn *et al.*, 1975) and animals (Edgar *et al.*, 1982).

Further consideration of the correlation analyses

Because the amount of plaque and tooth-brushing habits have some influence upon caries increment (Bellini, Arneberg and von der Fehr, 1981) and poor plaque control and poor dietary habits might occur in the same individuals, it was important to include these possible confounding factors in the analyses. The positive correlation between plaque level (as measured by G.I. and toothbrushing) and caries increment could not explain the relationship between caries and weight of sugars intake as the partial correlation controlling the data for G.I. or toothbrushing was similar to the bivariate correlation.

It was unexpected that the weight of sugary foods tended to be more highly correlated than the frequency of consumption (Table 3). This was not because the units of frequency of eating were small integer numbers as, for each subject, they were averaged over 15 days. Differences between correlation coefficients were slight and never statistically significant; nevertheless, the trend was towards higher correlations with weight than with frequency. We can give no explanation for this finding, which is at variance with other diet and caries evidence.

On average, 63 per cent of sugars intake was consumed as snacks, compared with 45 per cent of energy and 22 per cent protein. The importance of snack foods compared with total daily intake can be seen in Table 4 where, except for all sugared drinks, the correlation coefficients were higher and more were statistically significant when only snack foods were considered.

Information on the importance of concentration of sugars in a food is limited. Our data support the idea that concentration of sugars is an important variable but, because of the low correlations we found, it is not possible to estimate a threshold concentration value above which the food or dietary item might be considered potentially harmful.

The negative correlation between caries increment and the interval between eating a food containing more than 10 per cent sugars at bedtimes (Table 3) supports the view that eating sugary foods shortly before going to bed is likely to be particularly harmful (Palmer, 1971). When the data were controlled for G.I., the partial correlation was slightly higher (-0.118, $p = 0.02$) suggesting that levels of plaque control were not the explanation. However, the total amount of sugar consumed per day was an important factor because, when the data were controlled for weight of total sugars, the partial correlation fell to -0.032 ($p = 0.5$). Thus, it seems that the total daily intake of sugars is a more important variable in caries aetiology than the consumption of sugars shortly before bed.

Two unexpected statistically significant correlations were observed. First, that milk intake was positively correlated with caries increment (Table 3). Although cow's milk contains 4 per cent lactose, lactose is considered to be less cariogenic than sucrose, glucose or fructose (Frostell, 1970; Imfeld, 1977) and the high Ca and P levels are believed to protect the enamel from dissolution (Jenkins and Ferguson, 1966). There was a tendency for milk intake to be associated with sugar intake, for, when partial correlation analyses were performed controlling for sugars minus lactose, the correlation fell and, although no longer statistically significant, was still positive. The partial correlation was virtually unchanged ($+0.085$) when the data were also controlled for G.I. Secondly there was a correlation between caries increment and vitamin *D* intake (-0.117, $p = 0.009$) which was observed in the boys (-0.238, $p = 0.0004$) but not in the girls ($+0.006$, $p = 0.5$). The pre- and post-eruptive caries-preventive effect of vitamin *D*, noted by Mellanby and her co-workers (Young, 1937) were in both sexes.

Although we consider our longitudinal study to be superior to the more common cross-sectional type, the falling prevalence of dental caries raises doubts

about the optimum length of such a study. It is likely that three years should be the minimum. Increasing the number of days during which diet is assessed would increase the reliability, and would be achieved more efficiently by increasing the number of diet surveys per subject rather than the number of days per survey (Hackett *et al.*, 1983). Our use of multivariate analyses was of value in ranking dietary habits and investigating the influence of confounding factors but, because of intra-subject variation in diet intake in particular, was not suitable for estimating the importance of dietary habits in caries aetiology. Comparison of caries development or dietary habits between groups of subjects was of greater assistance, and affirmed the importance of dietary sugar. Evidence upon which dietary advice is based comes from many sources and it is important that longitudinal studies in human populations are undertaken in order to enhance this evidence.

Acknowledgements—The study was funded by an M.R.C. Project Grant. We would like to thank Miss Alison Black for advice on planning the dietary assessment, Mr A. D. French and Mr R. S. Ferrell for assistance in the planning and conduct of the survey; Mr D. J. Lovelock and the staff of the Department of Radiology, Newcastle Dental School for processing radiographic films; the Northumberland Health and Education authorities and schools for facilitating the study, and the children and their families for participating so conscientiously and cheerfully.

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Caries Res. 1987;21:464–473

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0008–6568/87/0215–0464 $ 2.75/0

Relative Cariogenicity of Starch and Sugars in a 2-Year Longitudinal Study of 405 English Schoolchildren

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Key Words. Caries, human · Diet · Epidemiology · Starch · Sugars, dietary

Abstract. Data from a longitudinal, observational study of diet and dental caries were analysed to assess the relative cariogenicity of starch and sugars. The correlation between 2-year DMFS caries increment and weight of daily intake of sugars was positive and statistically significant, while the corresponding correlation for starch was weakly negative after controlling for confounding variables. The difference in the possible relative cariogenicity of starch and sugars occurred, however, almost entirely in fissure surfaces and was not observed in approximal surfaces. Children (n = 27) with high starch/low sugars intake had lower caries increments than children (n = 32) with low starch/high sugars intake, but these differences were not statistically significant. The high-starch/low-sugars eaters consumed more energy but ate less frequently than the low-starch/high-sugars eaters. However, partial correlation analyses revealed that differences in frequency of eating could not explain the trend towards a stronger relation between sugar intake and caries than between starch intake and caries development.

The cariogenicity of dietary sugars is well documented [Newbrun, 1983; Rugg-Gunn, 1983], but the cariogenicity of dietary starch has received less attention [Krasse, 1982].

In animal experiments, raw starch has low cariogenicity [Grenby, 1970; Havenaar et al., 1984], but it has been known for some time that fine rolling and heating of starch alters its molecular composition [Thomson and Wills, 1976] and increases its cariogenicity [Grenby, 1967; Green and Hartles, 1967a, b]. Bread [König, 1969] and starch [Bowen et al., 1980] cause less caries than sugar, but mixtures of starch and sugar, such as in biscuits, have been shown to be cariogenic [Grenby and Bull, 1977].

Plaque pH experiments have indicated that the acidogenicity of raw starch is low [Frostell, 1972], but more recent experiments using indwelling glass pH elec-

trodes have suggested that the acidogenicity of cooked starch solutions and white bread are similar to those of sugar solutions or sugary foods [Schachtele and Jensen, 1981; Mörmann and Mühlemann, 1981; Jensen and Schachtele, 1983].

Epidemiological data from observational and interventional studies have, on the whole, suggested that dietary starch is associated with low caries development. Sreebny [1982] found a positive correlation ($r = +0.72$) between the availability of sugar and DMFT in 12-year-old children in 47 countries. He found a negative correlation ($r = -0.25$) between grain availability and DMFT in these same countries [Sreebny, 1983], although for wheat alone the correlation with DMFT was positive ($r = +0.45$). Children living in Hopewood House [Harris, 1963], patients with hereditary fructose intolerance [Newbrun et al., 1980], and subjects in the Turku sugar studies [Scheinin and Makinen, 1975] all consumed low-sugar, high-starch diets, and developed little or no caries. However, Mörmann and Mühlemann [1981] have questioned the applicability of these findings, since such dramatic reductions in sugar are unlikely to occur in modern western society and the combination of starch and sugar in our diet may be as cariogenic as sugar alone.

In order to investigate the relation between caries, and dietary starch and sugars, data from a recent longitudinal observational study were examined: findings of the relation between caries and sugars intake have been published [Rugg-Gunn et al., 1984]. The subjects were young adolescents who consumed a relatively unrestricted diet which could be described as a modern western diet.

Materials and Methods

The design, conduct and method of data analysis have been described previously [Rugg-Gunn et al., 1984]. Briefly, 466 children living in south Northumberland (water $F^- < 0.01$ mg/l), England, and initially aged 11–12 years, volunteered to participate in a 2-year observational study of diet and dental caries. They received an annual examination for caries (including bite-wing radiographs) and gingivitis by one dentist, and recorded their diet on five occasions during the 2 years using a 3-day diary with interview on the 4th day from one dietitian. The 2-year caries increments were the differences between the first and third annual examinations, and the gingival index used in data analyses was the average of that recorded at these two examinations. The dietary information was converted into nutrient intake (mean of the 15 days for each subject) using food tables [Paul and Southgate, 1978]. The validity of the dietary data collection method was measured by (a) comparing nitrogen input recorded in the diary with nitrogen output, and (b) comparing records of weighed meals with descriptions in the diaries [Hackett et al., 1983]. The reliability was measured by examination of between- and within-subject variances and was between 0.75 and 0.80 [Hackett et al., 1983]. In the food tables, sugars content was total sugars including sugars occurring naturally. Weight of starch was obtained by subtracting weight of sugars from weight of available carbohydrate. Frequency of intake of all foods and drinks was recorded: separate intakes being distanced from a previous meal by 30 min, and from a previous snack by 15 min [Rugg-Gunn et al., 1984]. These times were chosen because plaque pH begins to rise about 15 min after ingestion of sugar, and it was thought likely that eating a meal would take about 15 min longer than eating a snack.

In the present investigation, Pearson's correlation coefficients were calculated to rank the dietary variables according to the strength of their association with caries increment. The effect of possible confounding factors was investigated by partial correlation analysis [Snedecor and Cochran, 1967].

The caries increment of the two extreme groups of subjects was compared: the groups were subjects with (i) high starch, low sugar intake, (ii) low starch, high sugar intake, separated by the 65/35 percentiles. These percentiles were the same for starch intake (g) and sugar intake (g) and chosen to give approxi-

mately 30 subjects in each group. Differences in caries increments between the groups were tested using Student's t test. Two-tail tests of statistical significance were used throughout.

Results

405 children (193 boys, 212 girls) completed all aspects of this study. Their mean age, 2-year caries increments, gingival index and dietary intakes are given in table I. Most (57%) of the caries increment occurred in fissures, 36% occurred in approximal surfaces and only 7% in free smooth surfaces. The mean baseline DMFS was 5.00 and the mean final DMFS was 8.63, representing a mean increment of 3.63. The sum of the increments for the three types of tooth surface is higher than the whole mouth DMFS increment because some surfaces (e.g. buccal surfaces of lower molars) can exhibit both fissure and free smooth surface lesions. Starch comprised 56% of the carbohydrate intake (table I).

Table I. Mean (± SD) age at baseline, 2-year caries increments (four indices), gingival index (mean of baseline and 2nd-year examinations), and dietary intake of carbohydrate, sugars and starch, as well as energy intake and frequency of intake in 405 12- to 14-year-old children

	Mean	SD
Age	11 years 7 months	3.5 months
Δ DMFS	3.63	3.58
Δ DFS (FS)	2.10	2.01
Δ DFS (SS)	0.24	0.65
Δ DFS (AP)	1.34	2.13
Gingival index	0.80	0.36
Carbohydrate, g/day	271.7	50.7
Sugars, g/day	118.4	29.4
Starch, g/day	153.4	31.8
Energy, MJ/day	8.9	1.5
Frequency of intake times/day	6.8	1.8

FS = Pit and fissure surfaces; SS = free smooth surfaces; AP = approximal surfaces.

The bivariate correlation between DMFS increment and sugars intake was +0.105 ($p < 0.05$; table II). This was little altered when it was controlled for starch intake ($r = +0.106$, $p < 0.05$). Conversely, the low positive correlation between DMFS increment and starch (+0.019, $p > 0.05$) became negative (-0.022, $p > 0.05$) when the correlation was controlled for intake of sugars. Caries increment in pit and fissure surfaces showed the same pattern as total DMFS increment, giving a partial correlation with sugars intake, controlling for starch, of +0.149 ($p < 0.005$), while the partial correlation with starch intake, controlling for sugars, was -0.043 ($p > 0.05$). The correlation between caries increments in smooth and approximal surfaces, and starch and sugars intakes were not statistically significant, and were variable in direction (table II). The correlation between sugars and starch intake was +0.37 (fig. 1). The significance levels were not changed by using logarithmic transformation of caries increment measurements to correct for skewness.

The delineation of the two extreme groups (high-starch/low-sugars, low-starch/high-sugars) is shown in figure 1. The high-starch/low-sugars group had starch intakes which were over 164 g/day and sugars intakes less than 103 g/day.

Table II. Correlations between 2-year caries increments and sugars or starch intake (g/day) in 405 12- to 14-year-old children

		Bivariate correlations	Partial correlations controlling for	
			starch	sugars
Δ DMFS	vs. sugars	+ 0.105*	+ 0.106*	–
	vs. starch	+ 0.019	–	– 0.022
Δ DFS (FS)	vs. sugars	+ 0.143***	+ 0.149***	–
	vs. starch	+ 0.014	–	– 0.043
Δ DFS (SS)	vs. sugars	– 0.010	+ 0.009	–
	vs. starch	– 0.051	–	– 0.050
Δ DFS (AP)	vs. sugars	+ 0.042	+ 0.033	–
	vs. starch	+ 0.031	–	+ 0.016

Bivariate correlations are given, as well as partial correlations controlling for starch or sugars intake.
* $p < 0.05$, *** $p < 0.005$.

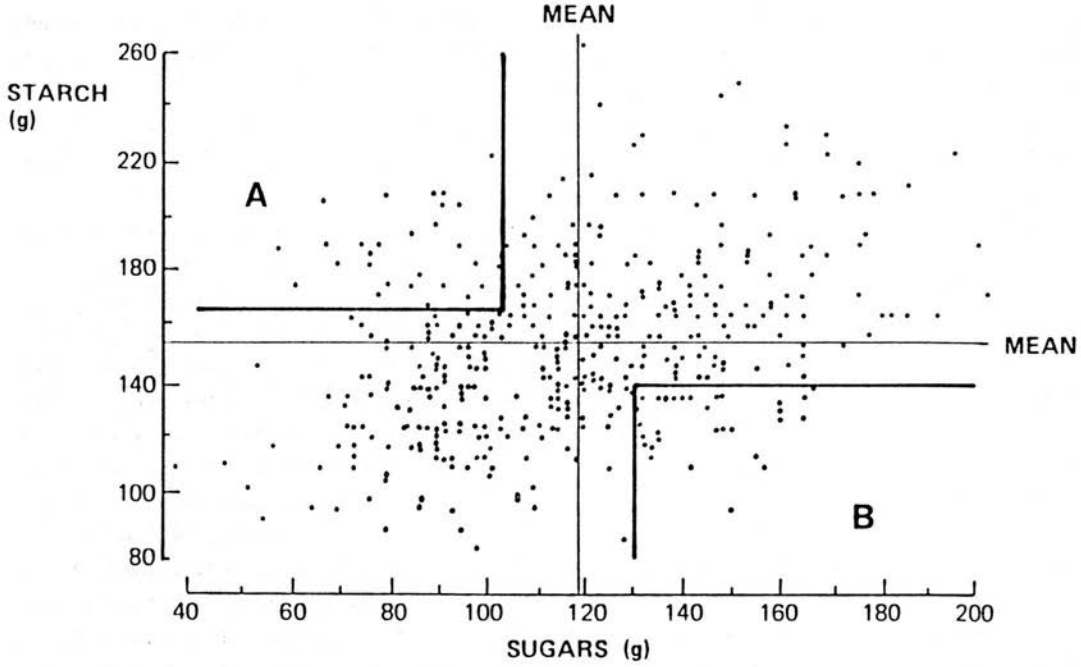

Fig. 1. Plot of mean daily intake (g) of sugars versus starch, for 405 subjects. The mean sugars intake (118.4 g/day) and the mean starch intake (153.4 g/day) are indicated, as well as the delineation, by 65/35 percentiles, of the high-starch/low-sugars subjects (A) and the low-starch/high-sugars subjects (B).

Table III. Mean age, starch and sugars intake, 2-year caries increments, gingival index, energy intake and frequency of eating all foods and drinks, in subgroups of children who ate either high-starch/low-sugar or low-starch/high-sugar diets

	High-starch/ low-sugars diet	Low-starch/ high-sugars diet	p (two-tail)
Number of subjects	27	32	
Age	11 years 6.2 months	11 years 6.6 months	0.59
Starch intake, g/day	186.7	124.5	
Sugars intake, g/day	86.7	143.4	
Δ DMFS	2.8	4.1	0.23
Δ DFS (FS)	1.6	2.6	0.09
Δ DFS (SS)	0.2	0.2	0.76
Δ DFS (AP)	1.1	1.3	0.65
Gingival index	0.8	0.8	0.99
Energy intake, MJ/day	9.4	8.7	< 0.01
Frequency of eating, times/day	5.7	7.8	< 0.01

The low-starch/high-sugars group had starch intakes which were less than 139 g/day and sugars intakes which were greater than 129 g/day. There were 27 children in the former group and 32 in the latter group (table III). The high-starch/low-sugars group consumed, on average per day, 62.2 g of starch more, and 56.7 g of sugars less, than the low-starch/high-sugars group. There was no difference in their mean age, baseline caries experience or gingival index. The children in the low-starch/high-sugars group developed 1.3 DMFS (46%) more caries than the children in the high-starch/low-sugars group. Nearly all of this difference occured in fissure surfaces but none of the differences in caries increments was statistically significant. The use of logarithmic transformation to correct for skewness of data did not alter the levels of statistical significance. Children in the high-starch/low-sugars group consumed more energy, but ate less frequently, than the low-starch/high-sugars group children.

There was a difference in the sex distribution between these two groups: 18 males and 9 females in the high-starch/low-sugars group and 11 males, 21 females in the low-starch/high-sugars group ($\chi^2 = 6.11$, $p < 0.05$). However, the pattern of differences between groups in frequency of eating, caries increment, gingival index and energy intake were similar for each sex separately as for both sexes combined. Despite small numbers of subjects, for each sex separately, the differences between groups in frequency of eating were statistically significant ($p < 0.05$). The difference in energy intake between the groups was statistically significant in the males (9.5 compared with 8.7 MJ/day, $p < 0.01$), but this difference between groups failed to reach statistical signifi-

cance in the girls (9.2 and 8.7 MJ/day, $p > 0.05$).

Because of the possible influence of confounding factors on the findings of this observational study, further partial correlations were calculated (table IV). These factors were age, sex, gingival index, frequency of eating and starch (for the caries vs. sugars correlations) and sugars (for the caries vs. starch correlations). These partial correlations (table IV) were little different from those given in the two right-hand columns of table II, although the correlations between DMFS and sugars intake no longer reached statistical significance ($p = 0.09$). As in table II, the fissure surfaces showed the greatest difference in correlation between caries increment and sugars or starch intake.

The same six dependent variables (age, sex, gingival index, frequency of eating, sugars and starch intake) were entered into a multiple regression analysis with DMFS as the independent variable. For an increase in sugars intake of 30 g (approximately one standard deviation), caries rose by 0.36 DMFS, with a 95% confidence range of -0.07 to + 0.80 DMFS. For an increase in starch intake of 30 g (also about one standard deviation), caries increment decreased by 0.17 DMFS, with a 95% confidence range of -0.54 to + 0.20 DMFS.

Table IV. Partial correlations between 2-year caries increments and sugars or starch intake, controlling for five possible confounding variables: age, sex, gingival index, frequency of intake, and starch or sugars intake

		Partial correlations controlling for age, sex, gingival index, frequency of intake and	
		starch	sugars
Δ DMFS	vs. sugars	+ 0.082	–
	vs. starch	–	– 0.045
Δ DFS (FS)	vs. sugars	+ 0.142***	–
	vs. starch	–	– 0.060
Δ DFS (SS)	vs. sugars	+ 0.023	–
	vs. starch	–	– 0.068
Δ DFS (AP)	vs. sugars	– 0.010	–
	vs. starch	–	– 0.002

*** $p < 0.005$.

Discussion

This is the first report of a longitudinal observational study of the relation between caries, sugar and starch. Such studies are difficult and expensive to conduct but, unlike animal, plaque pH, and other in vitro experiments, they give information on cariogenicity of foods in human subjects eating voluntary diets. It is important, therefore, to gain as much information from them as possible.

The merits of the longitudinal design and the optimum length for a study such as this have been discussed previously [Rugg-Gunn et al., 1984]. It was also pointed out that although correlation analysis is useful at ranking foods or eating habits in order of importance in caries aetiology, the size of the correlation should not be taken as a measure of the strength

of relationships since many factors act to depress the numerical value of the correlation. Comparison of caries increments of subjects at the ends of the frequency distributions of dietary variables is a better way to assess the clinical importance of dietary items.

In this respect, it should be noted that the composition of the two extreme groups in the present report was different from the composition of the two extreme groups described previously [Rugg-Gunn et al., 1984] since, in the present study, the groups were determined on the basis of both starch and sugars intake, rather than just sugars intake. This explains the differences in numbers of subjects and the caries increments in the groups in this, compared with the previous study. The groups in the present study were less extreme in their sugars intake, which is a likely explanation as to why none of the differences in caries increments between the 'high' and 'low' groups reached statistical significance in this study, but did in the previous study. It can be seen (table III, fig. 1) that the mean intake for both dietary variables differed between the two extreme groups by only two standard deviations (table I). It was difficult in this survey, which involved 405 subjects, to identify large numbers of subjects with very different diets. Making the groups more extreme in starch and sugar intake would have reduced the number of subjects per group and reduced further the power of the experiment to detect differences in caries increment. Conversely, increasing the number of subjects per group would have increased the similarity of their diets. The target of 30 as a group size was a compromise between these two aspects.

Using these data, further calculations were made to determine (a) the number of subjects required, in the extreme groups and in the total sample, in order to have a power of 95% of detecting the observed difference of 1.3 DMFS increment at the 5% level of significance, and (b) the size of the difference in caries increment between the groups that would be detected ($2\alpha = \beta = 0.05$) given the present sample size of 405 subjects. This information is of value to planners of similar studies. In answer to the first question, 268 subjects would be required per group (compared with 30 at present), and hence 3,620 in the whole sample. For the second question, given the present sample size, a difference of at least 3.6 DMFS increment between groups would be necessary to reach statistical significance at the 5% level. This would have meant the mean DMFS increments for the groups would have had to be, for example, 1.7 and 5.3, respectively.

In any observational study, the observed relation between disease and possible causative factors can be affected by confounding variables. Partial correlation analysis is a valuable method of determining the influence of these confounding variables and was used in this study. The amount of dental plaque was considered to be one possible factor which might have influenced the relation between diet and caries development and, in this study, plaque quantity and the effectiveness of toothbrushing was measured by recording gingival index [Rugg-Gunn et al., 1984]. Other possible non-dietary confounding factors were age (although all the children were from the same school year) and sex, and these were also used in the partial correlation analysis. Exposure to fluoride

was not considered an important confounding factor in this study since all subjects received water with negligible fluoride concentration, nearly all subjects reported that they used a fluoride dentifrice, and the use of fluoride dietary supplements and other topical fluoride agents was virtually non-existent.

The results of this study showed that dietary sugars were likely to have been more cariogenic than dietary starch. This is indicated by the correlation/partial correlation analyses, and supported by the comparison of groups of subjects with extreme dietary intakes and multiple regression analysis. However, this conclusion should be made with caution since only some of the relationships were statistically significant. From both types of analysis, it appeared that the differences between the possible cariogenicity of starch and sugars occurred almost entirely in fissure surfaces, with little relationship observed in other surfaces. It is possible, though, that the low levels of caries occurring in the non-fissure surfaces might have masked the presence of a relationship (in either direction) since small increments and many zero scores depress the value of correlation coefficients [Appleton et al., 1986]. The suggestion in the results that approximal surfaces may be more susceptible to the cariogenic challenge of starch than fissure surfaces should be investigated further.

It should be noted that sugars intake embraced all dietary sugars. It could be argued that the main added sugar, sucrose, is the most cariogenic, but individual sugars were not listed separately in the food tables and it was not possible to investigate this point in this study.

The observation that the high-starch/low-sugars group children had a higher energy intake will interest nutritionists. It could not be explained by differences in age or sex. Measurement of energy output was not attempted, but the gain in body weight of the high-starch/low-sugars eaters (11.1 kg) during the 2 years was fairly similar to that (10.5 kg) in the low-starch/high-sugars eaters ($p = 0.5$). Likewise the mean Quetelet indices (a measure of obesity: weight/height^2) for the two groups at the final examination were similar (19.7 and 19.1, $p = 0.4$). This suggests that energy output was higher in the high-starch/low-sugars eaters.

The higher frequency of eating or drinking in the low-starch/high-sugars group children was also of interest. This was found in both sexes. Snacks have a higher sugar content than meals [Rugg-Gunn et al., 1984] so that it is not surprising that a higher frequency of eating occurred in the children who had the higher sugar intakes. Partial correlation analyses (table IV) revealed that differences in frequency of eating between the groups were unlikely to have been the explanation for the higher caries increments in the low-starch/high-sugars group. It is also interesting to note that rats fed high-sugar/low-starch diets tend to eat more frequently than rats fed low-sugar/high-starch diets [Hefti and Schmid, 1979].

Current nutritional guidelines in the UK [National Advisory Committee on Nutrition Education, 1983] advise a reduction in the consumption of sugar and an increase in the consumption of starch. The results of this study do not conflict with this advice. Walker [1984] highlighted the lack of studies investigating the effect that

this would have on dental health; it is important that such interventional dietary studies in human subjects are conducted.

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Reprinted from BRITISH DENTAL JOURNAL, Vol. 162, No. 9, pp. 335-340, 9 May, 1987

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Oral Biology

The water intake of 405 Northumbrian adolescents aged 12–14 years

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Br Dent J 1987; **162:** 335
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Four hundred and five children, initially aged 11–12 years and living in south Northumberland, recorded their intake of all foods and drinks for a total of 15 days during a 2-year period, 1979–1981. Total water intake was calculated for each day for each child using food tables. The mean water intake from all sources was 1259 grams per day (g/day) (SD 245; 5 and 95 percentiles, 892 and 1676). Sixty-six per cent of water came from drinks and 34% from foods; 515 g/day (SD 201) was consumer-added water and 101 g/day (SD 76) water added by manufacturers. Water intake was higher in the boys (1315 g/day) than in the girls (1209 g/day), but no marked differences in the sources of water were observed between sexes. There were only minor differences in intake between social classes. Water intake was slightly higher at weekends, but little variation was observed between seasons of the year. The variance of the mean water intake and the range of intakes was much less when 15-day mean intakes, rather than 24-hour measurements, were used in analyses, indicating that ranges of 24-hour measurements can be misleading. If a number of assumptions were made, the maximum mean intake of dietary fluoride (F) in these children was estimated to be 1·3 mg F/day, of which 0·6 mg F/day might come from fluoridated water.

Water is essential for life and can be an important dietary source of minerals; this in turn may be related to a number of diseases.[1] The inverse relation between the fluoride concentration in drinking water and the prevalence of dental caries is well known.[2] Despite the importance of dietary water, the lack of information on water intake in Britain was recently emphasised.[3]

Much of the information on water and fluoride ingestion upon which systemic fluoride therapy is based was obtained in the United States 40 years ago. Since then, changes have occurred which could affect the basis for current advice. First, energy intake has fallen steadily in the UK[4] (probably due to a more sedentary lifestyle) and this is likely to have been accompanied by a reduction in water intake; second, ingestion of fluoride from sources other than water may have increased.[5]

While recommended daily allowances for many nutrients are published periodically in Britain,[6] water is not one of those listed. One of the few countries to do so is West Germany, where the German Society for Nutrition[7] have published 'accepted values'. For example, for 10–12-year-old children, the figure is 70–85 ml/kg body weight/day (about 2500 ml/day). This includes water in food but excludes metabolic water (water produced by metabolic oxidation in the body).

The early American estimates of water intake used figures published by Adolph[8] who, in a lengthy discussion, suggested that: 'Roughly a convenient liberal standard of total water intake for any mammal is 1 cc per Calorie'. From this, McClure[9] estimated that 10–12-year-old children would have a water requirement of 2500 ml (including water from food), of which about 1000 ml could come from the tap. If the tap-water contained 1 ppm of fluoride, then the child would ingest 1 mg F/day from this source. These figures appear to have formed the basis for subsequent dosage schedules for fluoride dietary supplements.[10]

One of the first epidemiological studies of water intake was undertaken by Galagan *et al.*[11] on 455 children living in two Californian communities. The 'fluid' intake (averaged over summer and winter) of the oldest children (9–10 years) was about 1500 ml, of which about 760 ml came from the tap. This study was repeated in Edmonton, Canada, by McPhail and Zacherl,[12] who reported that the total fluid intake of 9–10-year-olds was 1310 ml, with tap-water providing 676 ml. In both studies, milk accounted for most of the non-tap-water fluids.

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Oral Biology

Two further North American studies were restricted to young children. In the first, children under the age of 6 years, living on Long Island New York, were reported to consume about 294 ml of tap-water/day.[13] This is similar to the figure of 312 ml of tap-water/day found in the second study of 797 children aged 30–36 months living in four dissimilar geographical areas of the United States.[14]

In Britain, there have been few epidemiological studies of water intake. Longwell[15] reported the results of a pilot study undertaken by Bransby on 270 inhabitants of Willesden, London. The average daily 'fluid' intake was 1944 ml for men, 1415 ml for housewives, and 1029 ml for 5–14-year-old children. For the children, 500 ml came from the tap, 443 ml from milk and 86 ml from soft drinks.

The second, more recent British study was undertaken by the Water Research Centre.[16] Information was collected from 3564 people in England, Wales and Scotland. For the whole sample, the mean daily intake of all 'liquid' was 1589 ml, of which 955 ml was tap-water. In the 5–11-year-olds, the boys had a total liquid intake of 986 ml, with 550 ml coming from the tap, and the girls, 902 ml, of which 533 ml was tap-water. Among 12–17-year-olds, the corresponding figures were 1401 ml and 805 ml for the boys, and 1198 ml and 725 ml for the girls.

Crosby and Shepherd[17] investigated the fluid intake of three age-groups in Perth, Australia. The number of subjects in each group was small: 16 3–5-year-olds, 18 12–15-year-olds, and three adults. However, data were collected for each subject on many days during both summer and winter. In winter, the 12–15-year-old children had a mean fluid intake of 994 ml, of which about 568 ml came from the tap, while in summer, their fluid intake was 1395 ml, of which about 1059 ml came from the tap. When the daily intake was plotted against maximum daily air temperature, an increase in fluid intake was observed only when the temperature exceeded about 70°F (21°C).

Gillies and Paulin[1] reported that the mean tap-water intake of 109 New Zealand adults was 1250 ml per day (other fluids were not recorded), while in a recent abstract,[18] the mean daily fluid intake of 409 Canadian 12-year-olds was reported to average 1211 ml, of which 'less than 50%' was tap-water.

All the above epidemiological studies have estimated 'fluid' intake and have excluded water present in foods. They have also excluded any tap-water used in cooking and then consumed as part of a food. Very few studies have investigated water intake from all sources. From a study of the water balance in three young men, Passmore *et al.*[19] reported that for a young man with an energy intake of 8·8 MJ leading a sedentary life, the total daily water requirement was 2574 ml, of which 1115 ml came from solid food, 1180 ml from dietary liquids and 279 ml from metabolic water. In a brief report[20] of the water intake of 59 pregnant Cambridge women, a mean total water intake of 1958 ml/day was recorded; 590 ml came from food, 910 ml from the tap and 458 ml from milk and other non-tap-water drinks.

These published data suggest that the tap-water intake in young adolescents, for example, may be less than the one litre postulated by McClure[9] and provides less than the widely assumed dose of 1 mg F/day. Because of this, and the fact that there are no data on the intake of water from all sources in children, data from a recent dietary survey of young British adolescents have been analysed. These analyses have included a study of variations in intake in relation to sex, social class, season of the year and day of the week. The fluoride content of locally manufactured soft drinks was analysed so that tentative estimates of dietary fluoride intake could be made.

Materials and methods

Between September 1979 and July 1981, over 400 children initially aged 11–12 years recorded their entire dietary intake for 3 consecutive days on five separate occasions. These children were volunteers from seven middle schools in the Morpeth, Ashington and Newbiggin area of Northumberland. The fluoride content of the water supply to this area was $<0{\cdot}1$ mg F/l. The methods used[21,22] in the study and the analyses of the dietary,[23–26] dental,[27,28] growth[29] and social[30] data have been published elsewhere. Each child was asked to record, in a specially designed diary, a description of all foods and drinks taken, the approximate amount consumed in household measures and the time of consumption. They were then individually interviewed on the fourth day when the information was verified and, with the use of calibrated visual aids, a quantitative estimate of intake was obtained. The father's occupation was obtained and categorised by social class into groups I to VIII.[31]

Using computerised food tables[32] the data were converted into dietary variables for each of the 15 days for each child. Some updating of the food tables was required. Foods and drinks were categorised into a number of arbitrary groups (Table I) to determine the sources of dietary water. These were based on the item codes in the food tables and fell into two broad categories: drinks and foods. Soups were classed as foods. For non-carbonated soft drinks, the water content of the concentrated drinks was included in the non-carbonated soft drinks group and the water added to dilute it was included in the water group since this was the way these drinks were coded. For carbonated drinks and for infusions of tea and coffee, the water content was included within these groups. Milk in puddings (ie rice pudding, custard) was classed as a food and excluded from the milk group. The water content of soups, rice and pasta were as prepared for eating. The amount of 'consumer-added water' was estimated by summing the water content of groups: water as such, hot drinks made with water, and rice and pasta. The amount of 'manufacturer-added water' was estimated by adding the water content of carbonated and non-carbonated soft drinks.

For each subject, the mean intake over 15 days was used in data analysis, except in analyses by month of the year and day of the week. The months were grouped into four seasons of 3 months each, autumn being September to November. In comparisons of water intake on weekdays (Monday to Friday) with weekends, the mean weekday intake and the mean weekend intake were calculated for each child, to avoid assuming the independence of all 6075 (405×15) days in statistical testing; a paired *t*-test was then used. Analyses of variance of the total daily water intake were made in order to partition the variance between subjects, surveys, and days within surveys. These analyses were undertaken to estimate (1) the reliability coefficient of the water intake measurements and (2) the effect of increasing the number of days recorded per child on the frequency distribution of intakes and variance of the daily water intake.

The possible fluoride intake from water and drinks in these children was estimated assuming that consumer- and manufacturer-added water could range from zero to 1·0 mg F/l. An infusion of tea was assumed to contain 1·5 mg F/l,[33] in addition to the fluoride content of the water itself. Water supplies in the Newcastle area contain various levels of fluoride.[34] Carbonated drinks manufactured in four localities (Morpeth, Durham, Sunderland and Stanley) were bought from local shops and analysed for fluoride. The water supplies in the four areas were approximately 0·1, 0·5, 0·5 and 1·0 mg F/l, respectively. All types of containers (plastic or glass) were obtained where possible. Fluoride determinations were made in triplicate using an Orion fluoride ion-specific electrode after decarbonating the sample and adding Tisab buffer.[35]

Results

Four hundred and five children (193 boys, 212 girls) completed the 2-year study. Their mean age at the start was 11 years 7 months. The percentage of children in each social class group was: I+II, 21%; III, 43%; IV+V, 29%. Seven per cent were classed as miscellaneous (VI to VIII). Only one child was non-caucasian.

The mean daily energy intake for all children was 8·9 MJ, 9·5 MJ for boys and 8·5 MJ for girls. The mean water intake for all children was 1259 g/day (Table I). Sixty-six per cent of all water came from drinks and 34% from foods. Hot drinks provided approximately a quarter of the water intake, while milk, as a drink, provided 17%. Carbonated soft drinks gave only 78 g water per day, amounting to 6% of the total intake. Vegetables provided nearly three times more water than fruit. A negligible amount of water came from alcoholic drinks in these children. It was estimated that 101 g (8%) of all water was added by manufacturers, while a further 515 g (40%) was consumer-added. The remaining 643 g (52%) of the daily intake of water came from foods, milk and fruit juices. The variation in intake is shown by the 5 and 95 percentiles in Table I and the histograms of the intake of total water (fig. 1) and consumer-added water (fig. 2).

Total water consumption was higher in the boys than in the girls ($P<0·001$) (Table II). This was true for drinks and foods (both $P<0·001$). The weight of consumer- and manufacturer-added water was similar between sexes, but the proportion of total water from consumer-added water was higher in the girls (42%) than in the boys (39%) ($P<0·01$). Water from milk was higher in the boys (19%) than in the girls (16%) ($P<0·001$), expressed both in grams and as a proportion of total intake.

The water intake per unit of energy intake for all subjects was 0·14 g/kJ (SD 0·02); this is equivalent to 0·60 g/kcal (SD 0·10). The values for boys and girls were very similar: 0·14, 0·14 g/kJ, and 0·59, 0·60 g/kcal, respectively.

Differences between sexes were larger than differences between social classes. Data for social classes are not shown since the only statistically significant difference between social classes was observed for water from coffee, expressed both in grams and as a proportion of total intake. The amounts of water from coffee in social classes I+II, III and IV+V, respectively, were: boys 67 g, 115 g, 126 g; girls 95 g, 114 g and 171 g, per day.

Differences in water intake were observed between

Table I Water intake in 405 12-14-year-old Northumbrian adolescents, presented as mean (g/day), SD, 5 and 95 percentiles, and as percent of total daily water intake. These data were calculated using each subject's mean daily intake (averaged over 15 days)

Dietary sources	Mean	SD	Percentiles 5	Percentiles 95	Percentage of total water
All drinks	841	214	513	1227	66
Water	176	133	23	469	14
Hot drinks	336	226	6	741	26
Tea	214	204	0	641	16
Coffee and others	121	148	0	426	9
Soft drinks	109	80	17	250	9
Carbonated	78	75	0	208	6
Non-carbonated	22	23	0	68	2
Fruit juice	9	18	0	45	1
Milk	219	119	55	444	17
All foods	418	98	278	588	34
Vegetables	131	49	60	223	11
Fruit	44	33	3	108	4
Soups and gravy	42	31	3	105	3
Rice and pasta	4	6	0	18	0
Other	198	53	125	292	16
All sources	1259	245	892	1676	100
Consumer-added	515	201	189	871	40
Manufacturer-added	101	76	15	232	8

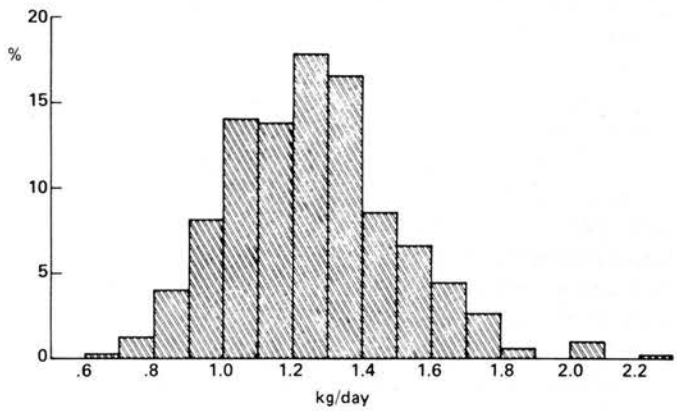

Fig. 1 Percentage frequency distribution of 405 adolescents according to mean daily total water intake (kg/day). Data were calculated using each subject's mean daily intake (which was averaged over 15 days).

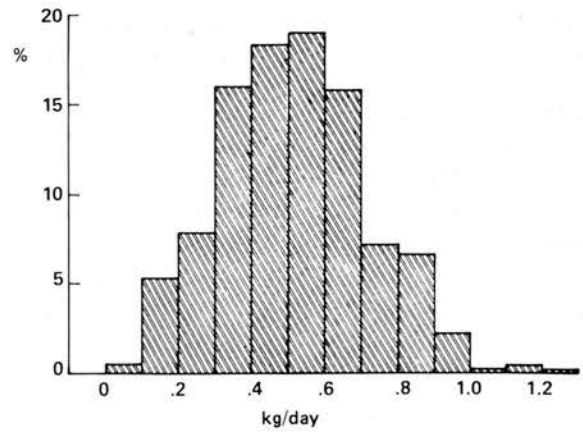

Fig. 2 Percentage frequency distribution of 405 adolescents according to mean daily intake of consumer-added water (kg/day). Data were calculated using each subject's mean daily intake (averaged over 15 days).

Table II Water intake (g/day) for 193 male and 212 female 12-14-year-old adolescents. These data were calculated using each subject's mean daily intake

	Males			Females		
Dietary sources	Mean	SD	Percentage of total	Mean	SD	Percentage of total
Drinks	876	209	66	809	213	66
Water	171	126	13	181	139	15
Hot drinks	342	235	25	330	217	26
Soft drinks	111	85	9	107	74	9
Milk	251	130	19	190	100	16
Foods	438	102	34	400	90	34
Vegetables and fruit	180	55	14	170	53	14
Soups and gravy	41	30	3	44	32	4
All sources	1315	238	100	1209	241	100
Consumer-added	517	208	39	515	196	42
Manufacturer-added	104	84	8	97	69	8

weekdays and weekends. Intake was higher on weekend days for total water (1242 g and 1292 g, respectively), hot drinks (324 g and 359 g) and soft drinks (95 g and 139 g), while consumer-added water intake was lower on weekend days (522 g and 504 g). All these differences were statistically significant ($P<0\cdot05$).

A lack of variation in water intake between the four seasons of each year for the 2 years is evident in figure 3. During this period the intake of water increased in these growing adolescents, but increased to a similar extent as energy intake.[23]

The results of the analyses of variance in the total daily water intake are shown in Table III. Using these figures the total variance can be apportioned in the following way: between children 27%, between surveys 20%, and between days 53%. The reliability coefficient of the estimates of each child's mean water intake was calculated to be 0·78 using a previously described method.[21] Much of the variation can be seen to be attributable to between days and surveys rather than between children, so that an increase in the number of surveys and days per survey decreased the variance of the sample mean intake. This is shown graphically in figure 4, where curve A, which represents the frequency distribution of 24-hour estimates of daily water intake, is flat; this indicates a large variation ($\sigma=415$). Curve B is the frequency distribution of the 405 15-day means. The standard deviation of the daily mean found is considerably less than for curve A ($\sigma=245$), since much of the between surveys and days variance has been removed. Curve C is very similar to curve B and is the theoretical curve where the only source of variation is between children ($\sigma=217$). While the range of the 24-hour intakes was 111–3910 g, the range of the 15-day mean intakes was 600–2245 g.

The mean fluoride concentrations in carbonated drinks manufactured in four local plants were: Morpeth 0·28 mg F/l, Durham 0·55 mg F/l, Sunderland 0·48 mg F/l, and Stanley 1·2 mg F/l. There was little difference in the fluoride concentration in cola and lemonade drinks, or between drinks in glass or plastic containers from the same manufacturer. Locally made drinks were not available in cans.

Discussion

The validity and reliability of the estimates of dietary intake in this study have been discussed elsewhere.[21,22] Energy

Table III Analysis of variation in the total daily intake of 405 children recorded in five surveys of 3 days each

	Sums of squares[a]	df	Mean square[a]
Between children	364·65	404	0·9026
Between surveys within children	315·28	1620	0·1946
Between days within surveys within children	367·43	4050	0·0907
Total	1047·37	6074	

[a] $\times 10^{-6}$

intakes are similar to those recorded in other British surveys of young adolescents.[22,36] The reliability of the estimates of energy intake was 0·79,[21] very similar to the present reliability coefficient for total water intake (0·78). A major source of methodological error in a study such as this is the use of food tables: food samples are likely to vary considerably in water content and estimating the water content of many foods is difficult.[32] The water content of drinks, however, will not be greatly affected. There is always a possibility of under-recording intake, although every effort was made to avoid this. When the volunteers were instructed on how to complete their diaries, 'a glass of water' was one of the examples given to emphasise the importance of recording all foods and drinks.

This is the first report of an epidemiological survey of total water intake in children. The recorded intake of 1259 g/day was about half the 2500 ml/day postulated by McClure[9] and proposed by the German Society for Nutrition,[7] for children. The mean total water intake in pregnant Cambridge women[20] (1958 g/day) was higher than the 1209 g/day recorded by our 12–14-year-old girls. Similarly, the intake of water from food by our girls (400 g/day) was lower than the 590 g/day reported for the Cambridge women,[20] and considerably less than the 1115 ml/day recorded by Passmore.[19] McClure[9] suggested that the total water intake should be 1 ml/kcal. Our figure of 0·60 ml/kcal is considerably below this theoretical estimate.

While the intake of 'fluids' by the children in our study was lower than those recorded in many of the studies listed in the Introduction, tap-water intake was fairly similar to other studies. Comparisons are complicated: not only by differences in age and climatic environment, but also because in some studies, only water from the tap in the child's home has been included, while any tap-water has been included in others. The mean daily tap-water intakes in our study (515 g from consumers plus 101 g from manufacturers) are roughly similar to those reported by Longwell[15] (500 ml), Crosby and Shepherd[17] (568 ml, in winter) and Clovis and Hargreaves[18] (about 600 ml), but are lower than those reported by Galagan *et al.*[11] (760 ml), McPhail and Zacherl[12] (676 ml) and Hopkin and Ellis[16] (725–805 ml for 12–17-year-olds) for children, and by Gillies and Paulin[1] (1250 ml) and Black and Paul[20] (910 g) for adults.

Our results, and the previously published data, indicate that, in children, the intake of fluoride from water containing 1 mg F/l, is likely to be considerably less than 1 mg F/day. This has been commented upon before[37] and there would seem to be a case for reassessing the dosage of fluoride dietary supplements which currently recommends 1 mg F/day for children over four years of age.[38]

The influence of environmental temperature on fluid intake is well known,[11,39,40] although the effect is likely to be

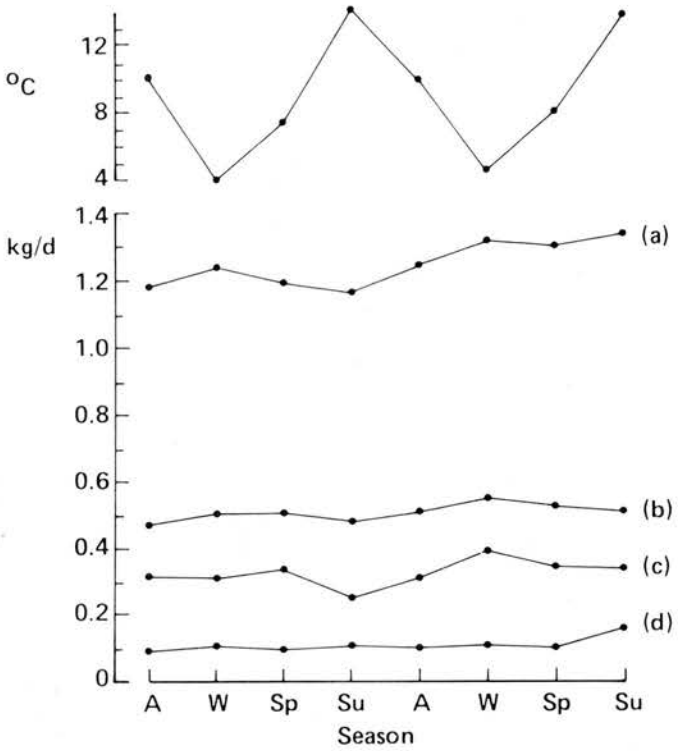

Fig. 3 Variation in water intake according to season of the year. The seasons are autumn (A), winter (W), spring (Sp), and summer (Su) for 2 consecutive years. The mean maximum daily air temperature (°C) in Newcastle for each season is given together with (a) total water, (b) all consumer-added water, (c) hot drinks, and (d) soft drinks intake. Data are for all 405 subjects.

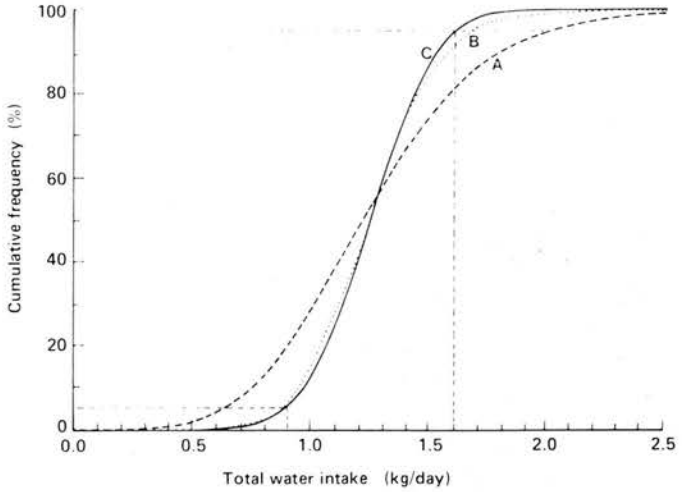

Fig. 4 Cumulative distribution curves of the total water intake (kg/day) for 405 children who each recorded their diet for 15 days. The mean intake was 1·259 kg/day. The three curves are: (A) when all 24-hour measurements (405 × 15) were analysed independently; (B) for the mean intakes (averaged over 15 days) for the 405 children; (C) the calculated mean intakes for the 405 children assuming that the number of days recorded per child was infinite and the within subject variance was zero. The 5 and 95 percentiles (cf Table I) are drawn.

small where air temperatures are less than 70°F.[17] The small seasonal variation in water intake observed in our study in Northumberland where, over the three summer months, maximum daily air temperature averaged less than 15°C (60°F), is in agreement with these earlier studies. Differences in water consumption were observed in our study between weekdays and weekends, similar in direction to those reported by Hopkin and Ellis;[16] this indicates the necessity of recording all days of the week in dietary surveys.

Ranges of nutrient intakes recorded in surveys are of interest and are often commented upon.[1] However, Black and Paul[20] have pointed out the importance of considering the effect of intra-subject variation upon these ranges. Dietary information is frequently obtained by the 24-hour recall method, although 7-day records have often been used in Britain. The dispersion of intakes is indicated both by the range of intakes and the variance. Both of these will be large when measurements are limited to 24 hours (fig. 4, curve A). The marked effect on the variance of increasing the number of days recorded per subject can be seen in figure 4 (curve B). Our method of recording the diet of each subject for 3 days on five separate occasions, provided a variance of mean intakes very close to the actual variance between subjects. These findings indicate the need for caution in interpreting the results of surveys obtained from 24-hour estimates of diet.

It was not the prime purpose of this study to investigate fluoride intake. However, it is possible to calculate likely intakes using various assumptions of fluoride levels in water, tea and food. If the fluoride concentration in tap-water had been 1 mg F/l, the mean weight of fluoride ingested from water would have been 0·515 mg F/day. Our investigations have indicated that carbonated soft drinks have approximately the same fluoride concentration as the public water supplies in the locality where the drinks were manufactured. If the soft drinks had been made in a fluoridated area, the mean fluoride intake in our subjects from this might have been about 0·12 mg F/day. The mean tea intake was 214 g/day. Assuming that an infusion of tea adds 1·5 mg F/l[33] to the water from which it is made, a mean of 0·321 mg F/day could have been ingested from this source. Thus, if these young adolescents lived in a fluoridated area and their soft drinks were made in a fluoridated area, their mean daily fluoride ingestion from drinks might have been about 0·96 mg F/day. There are no data on fluoride ingestion from foods in British children, but Singer *et al.*[41] reported that fluoride intake from foods by 15–19-year-old American males was about 0·33 mg F/day. This figure is likely to be higher than that for our adolescents, because of their lower energy intake compared with the young American adults. It would appear, therefore, that the mean fluoride intake from all dietary sources is unlikely to be greater than 1·3 mg F/day in young English adolescents.

Acknowledgements

The study on which these analyses were based, was funded by the MRC. The authors gratefully acknowledge the assistance of the Northumberland Education and Health Authorities, the schools, the children and the families involved.

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Printed in Great Britain by Bourne Offset Ltd., Iver, Bucks.

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Chronobiology International, Vol. 1, No. 2, pp. 97–101, 1984.
Printed in Great Britain.

0742-0528/84 $3.00+ 0.00
Pergamon Press

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THE DISTRIBUTION OF THE NUMBER OF MAXIMA IN A CYCLIC SEQUENCE

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Abstract—A simple statistical method is presented which tests for non-randomness in a cyclic sequence. The distribution of the statistic is tabulated for short sequences, and a large sample approximation is derived. Significant values of the statistic are given for sequences of length up to 52. Easily used computer programs to carry out appropriate calculations are presented.

Key words—Cyclic, circadian, seasonal, statistics.

Introduction

A very simple, but useful, statistical test of non-randomness in a sequence of observations is given by Kendall and Stuart (1). That is to count the turning points in the sequence, i.e. the number of times an observation is either greater than both of its neighbours, or less than them. If the sequence is cyclic this test can be applied with some modifications. By a cyclic sequence we mean one in which the last observation can be considered to be followed by the first, i.e. observations will have been collected over a multiple of an expected period of cyclic variability (a day, an oestrus cycle, a year, etc.). This article will derive the distribution of the test statistic m, the number of maxima in a cyclic sequence of n observations, and show how to use this distribution to test the hypothesis that variation in the sequence is due to chance, against the hypothesis that there is an underlying pattern which would tend to reduce the number of maxima. The distribution may be calculated exactly for sequences up to length 70 using a simple computer program given in the appendix. For longer sequences the distribution may be satisfactorily approximated; again a simple program is given.

The distribution of the test statistic

Let $p(m|n)$ be the probability of observing exactly m maxima in a random cyclic sequence of n observations. Then

$$n\,p(m|n+1) = 2m\,p(m|n) + (n+2-2m)\,p(m-1|n),$$

and from this recurrence relation and the fact that $p(1|3)=1$, and $p(0|n)=0$ for all n, we may calculate $p(m|n)$ for any values of m and n. To prove the recurrence relation consider a permutation of the first n integers; suppose that there are m maxima when the sequence is considered cyclically. If the $n+1$st integer is inserted in the sequence no new maxima will be created if it is adjacent to an already existing maximum: $2m$ of the n possible positions. Likewise if there are $m-1$ maxima this will be increased to m if the new member is inserted in any position not adjacent to these $m-1$ maxima: $n-2(m-1)$ of the n possible positions.

Table 1 uses the values of $p(m|n)$ for $n=3$ to 12 to calculate tail probabilities, i.e. the probability of observing m or fewer maxima in a random sequence of length n. From it we can see that observing only 1 maximum in a sequence of length 7 would be a significant departure from randomness at the 5% level (one-tailed) as would observing 2 maxima in a sequence of length 11. Table 2 gives the shortest length of sequence for which a given number of maxima is significantly lower than expected in a random sequence, for

Table 1. The probability of observing m or fewer maxima in a random cyclic sequence of n observations

Length of sequence n	Number of maxima m					
3	1					
4	0.6667	1				
5	0.3333	1				
6	0.1333	0.8667	1			
7	0.0444	0.6222	1			
8	0.0127	0.3746	0.9460	1		
9	0.0032	0.1937	0.8032	1		
10	0.0007	0.0878	0.6000	0.9781	1	
11	0.0001	0.0356	0.3951	0.9025	1	
12	0.0000	0.0130	0.2317	0.7641	0.9911	1

Table 2. Smallest number n of observations in a cyclic sequence for which the number of maxima m is significantly fewer than expected in a random sequence

m	Level of significance 0.05	0.01	0.001
1	7	9	10
2	11	13	15
3	15	17	19
4	18	20	23
5	22	24	27
6	25	27	30
7	28	31	34
8	32	34	38
9	35	38	41
10	38	41	45
11	42	45	48
12	45	48	52
13	48	51	
14	51		

sequence of length n up to 52. This is a more economical way of presenting the results than the more natural one of tabulating the critical values of m for each length of sequence, but a program in the appendix allows this to be done too.

Approximation for long sequences

When n is large it becomes computationally difficult to calculate the rows of Table 1, though the program in the appendix works for n up to 70. For n greater than 70 we may approximate the distribution of $p(m|n)$ by the Normal distribution. One may show, in a very similar fashion to Kendall and Stuart (1) that the expected value and the variance of $p(m|n)$ are

$$E\{p(m|n)\} = n/3$$
$$\text{var}\{p(m|n)\} = 2n/45.$$

This means that for an observed number of maxima m_0 we may treat $-(m_0 - n/3)/(2n/45)^{1/2}$ as approximately a Normal variate. It is interesting that a cyclic sequence yields simpler results than a non-cyclic one. Because the skewness of the distribution of $p(m|n)$ is $-0.23n^{-1/2}$ and the kurtosis is $-2.36n^{-1}$ we may expect the approximation to be quite adequate for n greater than 70. In fact for n=70 the approximation gives tail probabilities for 18, 19 and 20 maxima of 0.001, 0.007 and 0.029 compared with the actual values of 0.003, 0.014 and 0.053. This is indeed quite satisfactory, taking into account the rapid change in significance level which a change of 1 in m brings, though if the result is close to the critical value the exact probability should be calculated.

Example 1

Consider the following 3-hr estimates of cell birth-rate in the proximal small bowel of the normal rat given by Senior *et al.* (2):

Time (hr)	0900	1200	1500	1800	2100	2400	0300	0600
Birth rate (cells 1000^{-1} hr^{-1})	27.1	34.6	30.4	24.6	25.6	24.7	30.0	32.9

There are three maxima in this sequence (at 1200, 2100 and 0600 hr), so from Table 1 there is absolutely no evidence of a smooth rhythmic pattern in birth-rate.

Example 2

Consider the following 2-hr estimates of mitotic index in the proximal small bowel of the normal rat given by Al-Dewachi *et al.* (3):

Time	0900	1100	1300	1500	1700	1900	2100	2300	0100	0300	0500	0700
Mitotic index (%)	4.6	5.0	5.1	6.0	5.7	4.7	3.7	3.8	3.3	3.8	4.0	4.3

There are two maxima in this sequence (at 1500 and 2300 hr) and, from Table 1 or Table 2, this is significant at the 0.05 level. In other words this set of data provides significant evidence of non-randomness in the direction of a smooth function.

Example 3

The following data are simulated observations from an underlying curve

$$x = 20 + 10 \cos \pi (t-12)/12$$

Time (hr)	0900	1100	1300	1500	1700	1900	2100	2300	0100	0300	0500	0700
x_1	26.7	31.6	28.0	24.7	25.3	17.0	13.1	8.5	9.6	14.6	21.4	25.0
x_2	28.4	29.6	32.3	21.2	19.8	16.8	1.9	16.8	11.6	8.4	20.1	14.1

The sequence of x_1 values has 2 maxima (at 1100 and 1700 hr) and is therefore significantly non-random (P=0.013, one-tailed) whereas the sequence of x_2 values has three maxima (at 1300, 2300 and 0500 hr) and thus gives rise to a non-significant result (P=0.23, one-tailed). The difference between the sequences is that the x_1 were subjected to random Normal errors, mean zero and standard deviation 2 while the standard deviation of the errors in the x_2 sequence was 5. Greater errors of observation make it more difficult to detect non-randomness.

Discussion

The test described provides a simple way of looking for evidence of non-randomness in a cyclic sequence of observations made on a continuous scale. It makes no assumptions about the distribution of the variable, or about the exact nature of the non-randomness, though the one-tailed nature of the test assumes it arises from an underlying smooth, rhythmic process. The variation about this process is assumed to be uncorrelated between observations. If a model incorporating more information about these factors can be constructed (e.g. using differential equations), it will provide a better, more powerful method of analysis, but in its absence the test described here may prove of value.

The calculations involved for large samples may be onerous if exact probabilities are required, but the necessary computer programs can be written very concisely and should be easy to implement on a microcomputer.

No discussion of tied observations has been given; if ties between adjacent observations occur they can be broken by adding a small amount of random noise to the observations.

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Appendix

APL is a computer programming language which is easy to use and which is becoming available on an increasing number of microcomputers. We give here APL programs which perform certain tasks referred to in the text.

(i) To count the maxima in a cyclic sequence X.

```
    ∇ M←NMAX X
[1]   →(6≥⍴,X)/ERR,M←⍳0
[2]   →0,M←+/(X>1⌽X)∧(X>¯1⌽X)
[3]  ERR:'You must have at least 7 observations'
    ∇
```

(ii) To calculate the exact probabilities of 1, 2, . . . maxima in a cyclic sequence of length N.

```
    ∇ P←MAXIMA N;I
[1]   →((N≤6)∨(N>70))/ERR,P←⍳0
[2]   P←⍴,I←3
[3]  ⍝Line 4 implements the recurrence relation given in the text
[4]  L:P←(P≠0)/(P←((P×2×⍳⍴P),0)+0,P×((2|I)+2×⍴P)-2×⍳⍴P)÷I
[5]   →(N>I←I+1)/L
[6]   →0,P←+\P
[7]  ERR:'You must have between 7 and 70 observations'
    ∇
```

(iii) To calculate the Normal approximation probabilities of 1, 2, . . . maxima in a cyclic sequence of length N.

```
    ∇ P←MAXAPPROX N
[1]   P←NORMINT -((⍳⌊N-2)÷N-3)÷(N÷22.5)*0.5
    ∇
```

where NORMINT calculates the Normal probability from minus infinity to the vector of values Z (using Hastings' approximation).

```
    ∇ P←NORMINT Z;T
[1]   T←÷1+0.23164179×|Z
[2]   P←|((0.5×*¯0.5×Z×Z)×T×0.254829592+T×¯0.284496736+T×
      1.421413741×¯1.45315227+T×1.061405429)-Z<0
    ∇
```

(iv) To calculate the tail probability corresponding to the number of maxima in a cyclic sequence X. The Normal approximation will be used for sequences with over 70 observations.

```
    ∇ P←PROBMAX X;M
[1]   →(6≥⍴X)/ERR,P←⍳0
[2]   →(70<⍴X)/L
[3]   →0,P←(MAXIMA ⍴X)[NMAX X]
[4]  L:→0,P←(MAXAPPROX ⍴X)[NMAX]
[5]  ERR:'You must have at least 7 observations'
    ∇
```

(v) To calculate the largest number of maxima significantly lower, at levels S, than the expected value in a cyclic sequence of length N. The Normal approximation will be used for sequences with over 70 observations.

```
     ∇ M←S NSIG N
[1]    →(6≥N)/ERR,M←⍳0
[2]    →(70<N)/L
[3]    →0,M←+/(,S)∘.>MAXIMA N
[4]  L:→0,M←+/(,S)∘.>MAXAPPROX N
[5]  ERR:'You must have at least 7 observations'
     ∇
```

Examples of the use of programs (see Example 2 in the main text)

```
  SEQ← 4.6 5.0 5.1 6.0 5.7 4.7 3.7 3.8 3.3 3.8 4.0 4.3
  NMAX SEQ
2
```

(There are two maxima in the sequence.)

```
  MAXIMA 12
0.00003 0.01302 0.23169 0.76413 0.99114 1
```

(One maximum is highly significant, 2 give $P<0.05$.)

```
  PROBMAX SEQ
0.01302
```

(This does both the above calculations at once.)

```
  0.05 0.01 0.001 NSIG 12
2 1 1
```

(2 maxima gives $P<0.05$, 1 gives both $P<0.01$ and $P<0.001$.)

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Interpreting the Correlation Coefficient when One of the Variables is Discrete

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The effect on the correlation coefficient of discretizing data was investigated in two ways. First, the theoretical effect of dichotomizing data was calculated, and it was shown that the resulting correlation coefficient is considerably less than that between the underlying bivariate normally distributed variables. Second, computer simulations were performed of a model in which a continuous variable (measured with some error) gives rise to a counting variable through a mechanism in which the count is zero below a certain threshold value for the continuous variable and then increases linearly as the continuous variable increases. It was shown that the correlation coefficient between the observed values of the continuous and counting variables decreased as (a) the measurement error increased, (b) the slope of the relationship decreased, and (c) the number of counts decreased. It is concluded that caution is required when interpreting correlation coefficients when one or both of the variables consist of a few (say only four or five) discrete scores.

J Dent Res 65(11):1346–1348, November, 1986

Introduction.

Correlation analysis is used widely in biological research to investigate the strength and direction of relationships among variables. These variables may be measured in a number of ways: Some are continuous (*e.g.*, height, weight, daily intake of sugar, average number of daily intakes of sugar), some are counts (*e.g.*, number of intakes of sugar on a particular day, DMF index), and some are purely categorical (*e.g.*, sex, social class). Although the interpretation of the Pearson correlation coefficient between two variables requires both variables to be continuous, and indeed jointly to follow a bivariate normal distribution, some of the common dental variables, measured on a discrete scale, are often treated as if they were continuous. In the case of the DMF index, mean scores have in the past been high and their distribution approximately normal (Gaussian). However, with the decline in the prevalence and severity of caries, the numbers of scores recorded are fewer, the means are lower, and the distributions depart considerably from normality. The effects of using the correlation coefficient to investigate relationships involving such variables need study.

Rugg-Gunn *et al.* (1984) have presented correlation analyses in a report of a longitudinal observational study of diet and caries in 11-to-14-year-old children, and discussed the difficulty of interpreting their correlation coefficients when one of the variables (DMF) was discrete. The present study looks at the problem in two ways, first mathematically for a simple case, and second by means of computer simulation.

Methods.

The mathematical analysis of a simple case is examined first. Suppose that two variables, x_1 and x_2, are bivariately normally distributed with population correlation coefficient ρ. If they are each discretized into dichotomous variables on the basis of whether an observation is greater or less than the appropriate mean (or median) value, an association between them can be tested for by forming a 2×2 contingency table. Let the expected correlation coefficient from the dichotomized data be ρ^*.

By integrating the bivariate normal distribution function, it can be shown that the expected proportion of observations which are above the mean for both variables is $[1/(2\pi)] \cos^{-1}(-\rho)$, and hence that ρ^* is $(2/\pi) \sin^{-1}\rho$. This is always less than ρ, and indeed is approximately $2\rho/3$ for values of ρ less than 0.6 in magnitude (Fig. 1). This approximate result is given by Guilford (1965), who also discusses the situation when discretization occurs other than at the median. In our example, it can be seen that discretizing the two variables has considerably reduced the correlation coefficient which we expect to observe between them. A fuller description of the effect of discretization is given by Guilford (1965).

An alternative way of looking at this is to consider how many observations on the dichotomous variables would be required to give a 90% probability of detecting as significantly different, at the 5% level in a two-tailed test, two populations in which the proportion of occurrence of a particular feature was 0.4 in one and 0.6 in the other. To do this requires a sample of 282 (Casagrande *et al.*, 1978). But such data give rise to a correlation coefficient of 0.2, which is equivalent (because of the formula above) to a correlation coefficient of 0.31 in the underlying bivariate normal distribution. To test a sample correlation coefficient from such a population against zero (at the same significance level, and at the same power) requires 135 observations (Diem and Lentner, 1970). Again, it can be seen that discretizing the two variables has led to much greater difficulty (over twice as many subjects) in detecting a significant relationship between them.

The above example is of an extreme situation. More frequently the data are not dichotomous, but nevertheless can only be classified into discrete scores, for example when they arise as counts. The following example is not intended to model in

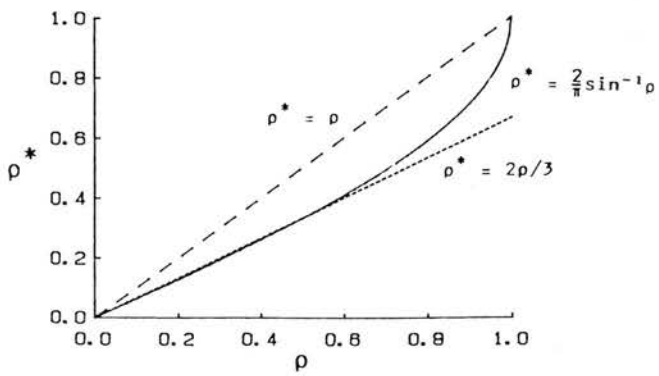

Fig. 1 — A graph of ρ^* versus ρ, where $\rho^* = (2/\pi)\sin^{-1}\rho$. This curve represents values from a 2×2 table. It can be seen that these values are always less than those obtained from the underlying continuous (normally distributed) data ($\rho^* = \rho$). The line for $\rho^* = 2\rho/3$ is also given.

Received for publication January 23, 1986
Accepted for publication July 22, 1986

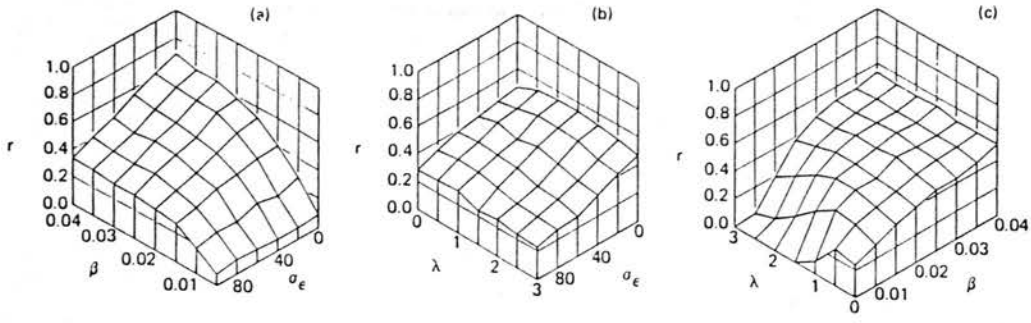

Fig. 2 — Isometric plots of the correlation coefficient r as a function of the parameters σ_ϵ, β, and λ of the model described in the text: (a) as a function of σ_ϵ and β, averaged over 10 replicates and the seven values of λ; (b) as a function of σ_ϵ and λ, averaged over 10 replicates and the eight values of β; and (c) as a function of β and λ, averaged over 10 replicates and the six values of σ_ϵ. Notice that the scales on the axes do not all run in the same direction. This is so that the shape of each relationship may be seen from the most suitable viewpoint.

any detail the relationship between caries increment and daily sugar intake (as an example of a continuous variable), but it may be helpful to consider it in the light of these variables.

Suppose that there is a continuous variable x which is normally distributed in the population with mean μ and standard deviation σ. In the present model this is sugar intake, with μ = 120 g/day and σ = 40 g/day. Suppose also that as a measurement of an underlying cariogenic factor, it is subject to error, either because of genuine random error in the method of measurement, or because of intra-subject variability, or because it itself is only associated with the real explanatory variable. This uncertainty, or error, may be modeled by a further normally distributed variate ξ with mean zero and standard deviation σ_ϵ. Suppose now that η, a multiple β times the sum of x and ξ, is used to create a new variate which has value zero if η is less than a value λ and otherwise is Poisson-distributed with mean $\beta(\eta-\lambda)$. This discrete variable y may be thought of as representing a count of caries, the count being large when β is large and small when it is small. A correlation between x and y has been built into the model.

In short, in the model of cariogenicity there is an underlying cariogenic factor which we measure imperfectly as x. If the factor is less than a threshold value λ, there will be no caries, but each increase of unity in x will cause the mean of a Poisson distribution, from which the number of carious teeth y arises, to increase by β. In the model, σ_ϵ represents noise, β represents the slope of the relationship, and λ represents a threshold value.

The relationship between the correlation coefficient (calculated in the usual way between x and y) and the parameters of the model, σ_ϵ, β, and λ, was investigated. Values used for each of the parameters were incremented in turn and were: σ_ϵ = 0(20)100, β = 0.005(0.005)0.04, and λ = 0(0.5)3, where a(h)b means "from a to b in steps of h". Programing was in APL, and a sample size of 50 was used throughout. The 336 different combinations of parameter values in each of the 10

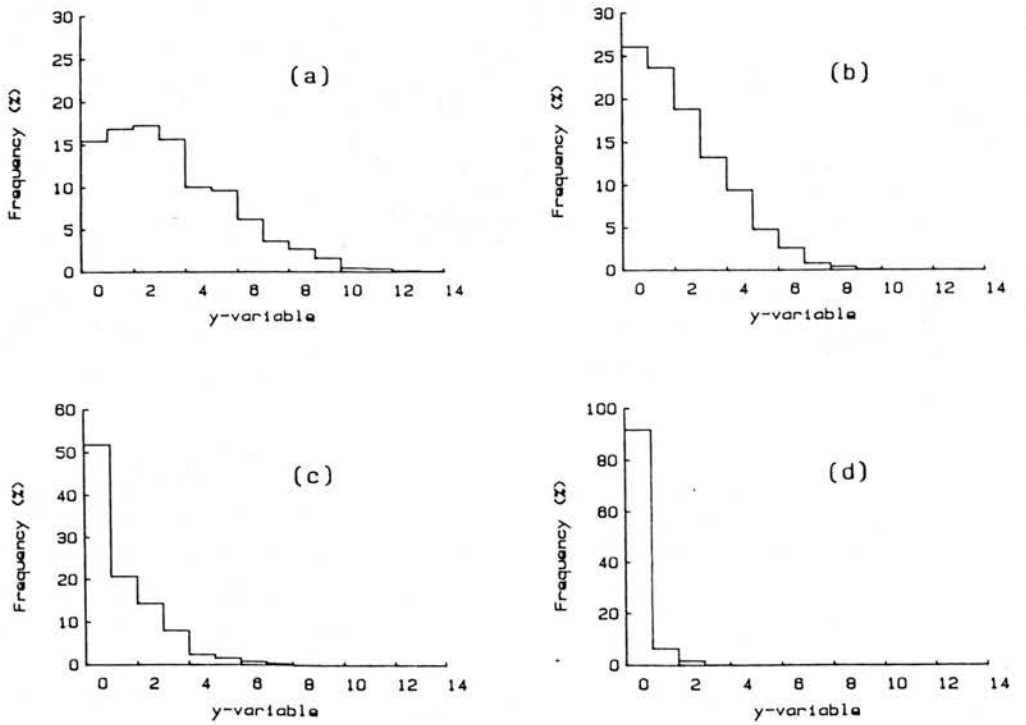

Fig. 3 — Histograms of the distribution of the counting variable y generated as described in the text, for the following parameters of the model:

	σ_ϵ (noise)	β (slope)	λ (threshold)
(a)	40	0.03	0.5
(b)	40	0.02	0.5
(c)	40	0.02	1.5
(d)	40	0.01	1.5

Notice that only graphs (a) and (b) have the same scale for the axis denoting percentage frequency.

replications used the same random numbers to generate values for x and ξ (and hence η), and all correlation coefficients were transformed using the z-transformation before being averaged (Diem and Lentner, 1970).

Results.

Each of the points in the isometric plots of correlation coefficient r as a function of the parameters σ_ϵ, β, and λ (given in Fig. 2) are averaged over the 10 replicates and the parameter, which does not appear in a particular graph. There are several points to note from the Figs. First, as σ_ϵ increases, the value of the correlation coefficient r decreases, particularly when β is high. This is to be expected: As the measurement of x reflects less well the underlying cariogenic factor, the correlation will decrease. Second, as β increases, the correlation coefficient increases. This is also perhaps to be expected: With continuous variables, the correlation coefficient increases with the slope. However, with continuous variables the correlation coefficient decreases as the variability in the y-variable increases, if the slope remains the same. In the present model, an increase in slope also causes an increase in variability in y (since the variance of a Poisson distribution has the same value as the mean), and this tends to reduce the correlation coefficient. Since the effect on the correlation coefficient of increasing the slope is proportional to the standard deviation of y (rather than its variance), the effect of the increasing variability is less strong than the effect of the increasing mean. Third, as λ increases, the correlation coefficient decreases, especially for low values of β. This is because, for large λ and low β, a very large proportion of the y values are zero, and the others are low counts, and, as has already been shown analytically, such discretization reduces the correlation coefficient.

Fig. 3 shows some distributions of y for different values of the parameters of the model. These are similar in general form to distributions of two-year DMFS, and DFS increments on fissure (FS), approximal (AX), and smooth (SS) surfaces, respectively (Rugg-Gunn *et al.*, 1984, and unpublished data). It should not be assumed that this model represents actuality, but the values of the parameters are reasonable. All four distributions used the same value for σ_ϵ — namely, 40 — and the slopes (β) and thresholds (λ) are in the correct relationship to one another [*i.e.*, when the value of η is over 0.5, there will be an effect on DMFS through DFS(FS) but not through DFS(AX) or DFS(SS)]. The corresponding correlation coefficients are, respectively, 0.54, 0.47, 0.42, and 0.41 — rather higher than those observed by Rugg-Gunn *et al.* (1984).

Discussion.

From the first (mathematical) investigation where the data were dichotomized, it can be seen that this extreme discretization of data leads to considerably smaller estimates of the correlation coefficient than would be obtained from the original continuous data. The second example, which is typical of the use of computer simulation to study a problem which cannot easily be tackled by purely mathematical techniques, extends this argument. In particular, it shows that the highest estimates of the correlation coefficient with another variable will be obtained for counting variables where large counts are possible. Thus, if sugar intake is correlated with several different measurements of caries, it cannot necessarily be assumed that the highest correlation coefficient is indicative of the strongest relationship: The distribution of the counting variable may have much to do with the values which are actually estimated. For example, fissure surfaces may show a higher correlation than approximal surfaces, but this may be because of the higher total numbers of surfaces and not because of any inherent propensity of fissure surfaces to have more caries than approximal surfaces. Indeed, in the study already cited (Rugg-Gunn *et al.*, 1984), the correlation between sugar intake (g/day) and caries was 0.143 for fissure caries (which represented 57% of all carious surfaces), 0.045 for posterior approximal caries (33% of carious surfaces), and 0.004 for anterior approximal caries (3%).

Similarly, caution is needed if a caries index is correlated with a continuous dietary variable (*e.g.*, daily weight of sugar intake) and a discrete one (*e.g.*, daily frequency of sugar intake). The distributions of the two variables may be such that even if frequency is the more important, it could exhibit the lower correlation coefficient.

Interpretation of the correlation coefficient is always difficult. Whenever possible, associations should be measured in terms of regression coefficients, and when correlation coefficients are studied in order to compare the strength of different relationships, results should be treated with great caution, especially if the distributions of the variables differ in shape.

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Reprinted from

[redacted]

PERGAMON PRESS
OXFORD NEW YORK LONDON PARIS

Opl Res. Q., Pergamon Press 1973. Vol. 24, pp. 19 to 25. Printed in Great Britain

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An Interactive Computer Model of University Room Usage

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An interactive computer model of room usage in a university was designed and constructed in order to study limitations imposed on student numbers by existing accommodation, to allocate rooms efficiently to classes and to plan new buildings.

INTRODUCTION

ALTHOUGH management interest in computer techniques is not new, universities have been slow to incorporate into their administration techniques proven in business and industry. Until recently only timetabling problems had been tackled in any detail on a computer, but some reports have now been given of models dealing with aspects of university resource allocation. An ambitious model of Fairfax University by Scarborough and Daniel[1] examines the general structure of the administration in an interactive model, without investigating any one problem in great depth. Longworth Smith[2] has described a mathematical model of the University of California at Berkeley which treats student demand for courses as a stochastic variable and obtains information on the required distribution of classroom sizes without any extensive validation of the model.

The problems of timetable construction and student scheduling in the context of the American University course structure have also been tackled by Holz[3] using the GASP program, and a further interesting development is the graph–theoretical approach of McDiarmid.[4]

APPROACH TO THE PROBLEM

Statement and limitations

In the session 1969–70 the University of Newcastle upon Tyne catered for some 6000 students. Of these, 4670 were in 39 departments outwith the Faculties of Medicine and Education. The University wished to determine whether lecture rooms available during that session would be sufficient to house an increased number of students; which departments could (by this criterion) accept more students; which variables in the system were critical; and the most appropriate sizes of any new rooms required.

The possibility of altering the existing class–teacher timetable was not considered, except in very special cases where a single class would otherwise necessitate the building of a new room.

Other important factors such as availability of staff, increased refectory facilities and extra student accommodation were not considered but could form parallel investigations. Laboratories, drawing offices and very small teaching rooms were not included in the study, since these rooms are frequently used by departments for specialized activities and may not be suitable for other purposes.

Advantages of an interactive model

A simulation of the system was chosen because it could produce results not easily obtainable by analytical or other methods, and also demonstrate the more complex interactions of the system thus helping the users of the model to understand it more fully.

Interaction between the computer and user frequently makes results more acceptable since it allows the use of personal experience and expertise. In the present instance the user need not have data available on all possibilities for reconstruction of rooms or division of classes as long as he can deal with specific cases when they arise.

An important practical decision is whether to treat the system as deterministic or probabilistic. The sizes of many classes are not known exactly until after the start of teaching and until then only estimates can be made. It would be possible to incorporate a random element into the model to take this uncertainty into account, but such a procedure entails considerable extra complexity without giving obvious compensating advantages. A practical reason for rejecting a stochastic model is that the need to replicate simulation runs is likely to occupy enough computer time to make interaction with the program infeasible, and so in the present situation it was decided to employ a deterministic model.

It was originally intended to simulate the system fairly closely by updating predicted student numbers year by year. This was found to be unnecessary in what was principally a long-term planning exercise, and the temporal aspect of the model became submerged.

A necessary part of any interactive study is the availability of suitable computer hardware. This exercise used an IBM 360/67 under the Michigan Terminal System (MTS), and programs were designed to be run from an IBM 2260 CRT display unit. Thus it was possible to obtain fast response times, to handle large quantities of data and to give the user quick but adequately intelligible requests and comments.

THE SYSTEM

Data organization

Lists were obtained from each University department of lectures given by its staff and to its students by other departments. For each lecture the time and

the room were given, and the number of students attending was broken down by the registration codes of the students.† (On registration at university each student is given a mnemonic code to describe his course for the year; there are 442 such codes.) For the computer model the fundamental unit is the lecture room, and so the data were arranged in the form of timetables of 35 weekly periods for the 115 rooms of capacity 20 or more which were considered.

A file of 14 pages (1 page = 4096 characters) was constructed to represent the timetables, each period containing the address (in another file) of a list of the registration codes of students in the room at that time, with the percentages of total students registered under these codes who were actually present. The file containing these data occupied 9 pages, and a file containing total numbers of students for each of the relevant codes, along with explanations of the codes, used 11 pages. A further 4 page file held the capacities of the rooms and descriptions of their locations.

Program organization

It is not necessary for the user of the suite of programs to understand the organization of programs or data, or any details of the MTS system. To initiate a session he issues a single command and thereafter responds to prompting and questioning. As a result of the initial command the CRT display is as shown in Table 1. The user's reply causes the relevant program to be loaded. The use of such a controlling program enables dynamic loading which reduces the size of the object module needed in the virtual memory at any time, an important consideration in a time-sharing environment. This advantage would be nullified if a great deal more loading were thereby entailed, but in fact each program, especially the main program, tends to be employed for a substantial time.

TABLE 1. CRT DISPLAY OF PROGRAMS AVAILABLE TO THE USER

Which program is required?
1. To perform reallocations
2. To print out timetables
3. To update student numbers
4. To add new rooms
5. To change existing rooms
6. To update the timetables
7. To gather statistics
8. To take no action

Enter the appropriate number

The programming language used throughout was ALGOL, and the compiled object modules occupy 39 pages; the total storage required for the system,

† I am extremely grateful to Dr. K. Mitchell for undertaking the considerable task of collecting and organizing these data.

excluding source programs and library modules, but including temporary space for updated files and line printer output is 142 pages. About half of this total storage may be required in the virtual memory at one time.

The suite includes programs which will investigate the room usage at a superficial level as a screening technique, run the main program, perform housekeeping on the data, or transform the data from symbolic form into readily comprehensible room-oriented timetables. The first option (Table 1) is the most important, but the other six programs are essential to its usefulness.

USING THE SYSTEM

An example terminal session

The first program called up by the user might be number 3. This would enable him to alter by some proposed ratio the student numbers in any specified departments or faculties. He would then run program 7 to gain an idea of the congestion in the rooms under investigation, through their percentage morning and afternoon utilizations and the frequency of overcrowding. If it seemed feasible he would alleviate this congestion by means of the main program (number 1: see Appendix); but it is possible that only the provision of a new room could cure it completely. Option 4 simulates the building of a room of the desired size and the user may then recall program 1 and conclude reallocations. The timetables may now be sent for printing using program 2, and while this is being done it is possible to store them (program 6) for future use. The user exits from the suite by calling option 8.

Many other permutations are, of course, possible, and the particular one chosen will depend to a high degree on the temperament and skill of the user as well as on the amount of congestion, the geography of the University and the number of rooms involved.

Experience gained in using the system

After the system had been tested on current student numbers and rooms, the effect of increasing the intake of one faculty (Arts) by 100 per cent was investigated. It was found that the simple reallocation algorithm used by the program was quite adequate, the user requiring to make relatively few allocations himself. The somewhat surprising result emerged that for such a large increase in students, given one change in the class–teacher timetable, only one new lecture theatre would be required, while several of the smaller rooms could be used for other purposes. It is unlikely that this finding would have been obtained without a computer model.

The timetables produced for the rooms and classes considered proved a satisfactory method of communication with those responsible for administration; these timetables were used to assess the amount of travelling involved in

the changes, and to ensure that no unacceptable congestion was likely in lifts or on staircases.

The use of the model demonstrated that time-sharing systems can lead to interaction in three basically similar but practically distinct ways. Firstly, a user may interact with a program, as in the allocation program, by issuing commands and responding to questions put by the program. Secondly, he may interact with a suite of programs, deciding which program is appropriate at a particular point in time; when to screen the data, to run the allocation program, perform housekeeping or run a program which will aid communication with other interested parties, such as the timetable printing program. Thirdly, the user may decide, possibly more slowly than at a terminal and in association with others, what his next course of action is to be. It is important that he should not feel bound to reply to the computer as quickly as it gives results and poses questions.

Future potential for the system

As well as continuing to employ the model in long-term planning situations it is hoped to use it to allocate lecture rooms to classes at the start of each university session. Registration codes are already stored on the computer, and such files could be used to provide an accurate breakdown of student numbers before lectures begin. Lectures which would be oversubscribed could be pin-pointed and alternative venues allocated before the situation occurred.

ACKNOWLEDGEMENTS

I am grateful to Professor E. S. Page for suggesting the exercise to me and for his helpful advice; and to Dr. K. Mitchell (Chairman of the advisory committee on the development plan) and Mr. J. Lynn (Consultant architect/planner) for making use of the model.

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APPENDIX

When the program detects that there are more students occupying a room than its official capacity it prints a message like:

"MONDAY AT 10
ROOM NO. 57 CAPACITY 40 44 STUDENTS."

No action is taken on this room until all further rooms have been dealt with for the same period; then, if the user is not dealing with all reallocations himself, the program takes the following course:

A. If the largest number of overcrowded students can be put in the largest empty room this is done. This step is repeated until it is impossible or no room is overcrowded.

B. If step A cannot be executed, then if the offending class can be exchanged with another so that both can be accommodated this is done and the program returns to step A. When neither step can be carried out the user is given control.

When control is passed to the user, either because he requested it or because the simple algorithm above has been unable to stop overcrowding, he has the capability of issuing the following commands, where the second form indicates the minimum necessary. The user has the choice of making his commands short or readily understood. (For clarity particular rooms and times are shown.)

I.	continue	*c*
II.	stop	*s*
III.	focus on room 12	*f* 12
IV.	does the situation occur again?	*d*
V.	when?	*w*
VI.	how many are in room 12 at 10, Friday?	*h* 12, 10, Fri
VII.	exchange classes in rooms 12 and 13	*ex* 12, 13
VIII.	put class in room 12 at 10, Friday in room 13	*p* 12, 10, Fri 13
IX.	list status	*l*
X.	repeat	*r*
XI.	move to 3, Friday	*m* 3, Fri

Further details of these commands will be supplied by the author on request. An example of their use is given in table 2.

TABLE 2. AN EXAMPLE OF THE REALLOCATION OF ROOMS BY THE USER

```
Wednesday at 10
  Room No. 41   Capacity 50   65 Students
  Room No. 47   Capacity 30   39 Students
The following rooms are empty and will take
the largest of these classes
  None
The following rooms are large enough
  Room No. 44   Capacity 70   55 Students
  Room No. 46   Capacity 80   76 Students
What action is required?
  List
Status of rooms on Wednesday at 10
  Room No. 41   Capacity 50   65 Students
  Room No. 42   Capacity 60   55 Students
  Room No. 43   Capacity 60   47 Students
  Room No. 44   Capacity 70   55 Students
  Room No. 45   Capacity 40    0 Students
  Room No. 46   Capacity 80   76 Students
  Room No. 47   Capacity 30   39 Students
```

TABLE 2. (*Cont.*)

```
What action is required?
  Ex 41,44
    Room No. 41   Capacity 50   55 Students
    Room No. 44   Capacity 70   65 Students
What action is required?
  Ex 41,43
    Room No. 41   Capacity 50   47 Students
    Room No. 43   Capacity 60   55 Students
What action is required?
  Put 47 at 10, Wed in 45
    Room No. 45   Capacity 40   39 Students
    Room No. 47   Capacity 30    0 Students
What action is required?
  Cont
```

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Reprinted from the BRITISH MEDICAL JOURNAL,
18 *February* 1978, **1**, 421–423

Choosing the programme for an international congress

D R APPLETON, D N S KERR

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British Medical Journal, 1978, **1**, 421-423

Summary and conclusions

The method of selecting abstracts for an international congress at which only 15% of submitted papers could be accepted entailed a panel of 12 assessors using their specialised knowledge, but presentation of the abstract was also important in selection. There was only a limited agreement between assessors in arranging abstracts in order of merit, so that a single assessor would be unacceptable.

Use of the full panel to grade all abstracts was very expensive, but it could be replaced, without unacceptable injustice, by dividing the work randomly among groups of three selectors.

Introduction

International congresses are often reviled and ridiculed. Some maintain that they exist solely for the benefit of the travel industry, but, as they continue to grow and proliferate, they probably meet some deeply felt need of scientists as well. At many the number of abstracts submitted for presentation grows from year to year; the programme is usually selected in one of two ways.

Selection procedures

The all-embracing approach—Plenary sessions are held in the

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morning with invited speakers. Submitted original papers are relegated to parallel sessions in the afternoons. Many delegates decide that if you are missing 13 sessions you might as well miss 14 and see the Taj Mahal/Uffizi Gallery/Golden Gate instead, so that these parallel sessions may be held in small seminar rooms, of which the modern conference centre has an almost limitless supply. Consequently there is little restriction on the number of abstracts accepted, and a small selection committee can quickly weed out the rubbish and divide the rest into convenient packets that fill the afternoons. This is tough on the last speaker, who addresses his wife and the previous speaker (if polite), but it ensures that most delegates get their travelling expenses, which allows the organisers to plan ahead and book attractive (and expensive) speakers for the mornings. This approach calls for good business acumen but presents few problems to the selection committee.

The selective approach—Submitted papers are given pride of place. They are delivered in plenary session and published. Their number is limited by the length of the meeting and the capacity of the unboggled human mind. Competition for a place on the podium is keen, its achievement an accolade, and in some societies a condition of full membership. Selection of papers must be fair and must produce the best programme possible.

We favour this approach but recognise its difficulties. It can work only if busy professionals, in their limited spare time, can read an abstract among several hundred and make a reliable judgment on it. Their task is made easier by the strict rules that are now enforced: abstracts must give explicit results and conclusions; promises that "the results will be discussed" are inadmissible; and they must be clearly typed in a stipulated space. None the less, an abstract that may have taken hours to write is read and judged in a couple of minutes. In an effort to increase reliability some societies use many assessors and pool their results but this is expensive in manpower. We have therefore analysed the selection exercise of a society using the selective approach to see how far it combines reliability with economy of effort.

Methods

The European Dialysis and Transplant Association (EDTA) holds an annual congress devoted to dialysis, transplantation, and general nephrology. The subdivision of time between these subjects is decided in advance. For the meeting at Helsinki in 1977, 329 abstracts were submitted (317 in time for this study) and 50 papers accepted. Authors were asked not to identify themselves in the text

and to send additional photostats of their abstract with the authors' names and institution obliterated. These anonymous abstracts were sent to 12 selectors who were members of the elected council and whose clinical and research interests spanned those of the society. Each marked abstracts on a six point scale from "0=reject" to "5=must accept" on the basis of scientific merit (defined as originality, adequate data, legitimate conclusions, and interest to the society). Selectors did not mark abstracts from their own institutions and for these the average of the other 11 marks was substituted in the selection process; the aggregate mark determined the paper's chance of acceptance until the appropriate section of the programme was filled. Nevertheless, for this statistical exercise most selectors kindly gave us confidential assessments of the papers from their own units, which we substituted for the average mark. In a few cases a selector did not mark an abstract, presumably because he felt unable to judge its contents.

To test the hypothesis that the presentation of an abstract is more important than its content, we submitted a duplicate set to a medical statistician with no specialised knowledge of nephrology (DRA) who marked them on presentation and general scientific approach. The 13 sets of marks were then analysed.

Results and discussion

HOW VARIABLE ARE SELECTORS?

Selectors are disappointingly variable, but not surprisingly so. The number of papers rejected varied from none to 23 and those marked "must accept" from none to 13. The mean mark varied from 1·85 to 3·12. This would not matter if selectors simply varied in the severity of their marking but arranged abstracts in much the same order over a similar scale.

DO THEY MARK OVER THE SAME SCALE?

No, the marks are not over the same scale, but the differences are less important than might be expected. The standard deviation of their marks varied from 0·83 to 1·22. Ideally the marks should be scaled to the same mean and standard deviation before aggregation but doing this altered only two of the top 25 and two of the top 50 papers in this study. Scaling the marks would present no problems to most university centres if they were received well before the meeting of the selection committee, but in real life this is unlikely to happen.

DO THEY ARRANGE ABSTRACTS IN THE SAME ORDER?

No, the abstracts are arranged differently. Seven abstracts received

0 from one or more selectors and 5 from another. There is, however, a significant correlation among the marks given by different selectors. We compared the marks of each official selector with the mean of the other 11 selectors for 288 abstracts on which we had a complete set of marks. The squares of the correlation coefficients (roughly the proportion of agreement) varied between $r^2 = 0{\cdot}05$ and $r^2 = 0{\cdot}29$. For this number of abstracts any value of r^2 over 0·023 is significantly different from zero at the 1 % level.

Each of the selectors was compared with all the others. The closest correlation ($r = 0{\cdot}41$) was between two physicians of the same nationality, background, and research interests. Not all the correlation coefficients were significantly different from zero, and the average was $r = 0{\cdot}20$. This may be compared with the work of Matthews and Leece[1] on impression marking—also on a 0-5 scale—of questions in A-level chemistry examinations; there the average correlation coefficient was 0·76. There was, however, an opportunity for bias in the latter study that did not exist in our own, and better agreement among examiners judging answers to the same question would be expected than among those assessing the relative merit of diverse research projects in a wide scientific field. The rather low correlation coefficients in this study probably represent what is achieved in programme selection and are a warning that the use of one single examiner, though commonplace in examination marking, is unlikely to prove suitable for selecting abstracts.

ARE PAPERS PICKED ON CONTENT OR PRESENTATION?

A cluster analysis (single linkage, using 1-r as the "distance" between two assessors) was performed, and the resulting dendogram is shown in the figure. Such a structure is a pictorial representation of the clusters being formed: those assessors who are most similar (5 and 6) join together first, then number 4 joins them, numbers 10 and 11 then form a separate group, and so on. There comes a point when the distance between individuals in different clusters is large enough for us to consider the clustering process to be finished. It would seem reasonable in this study to say that this occurs in the gap before the group containing assessors 1 and 2 joins the main group. The dendogram can therefore be interpreted as showing that 10 of the official selectors tended to group together, the other two being rather dissimilar from one another as well as from their colleagues. This may reflect their respective interest in two of the topics that are of deep concern only to a minority of the society, as reflected in the abstracts submitted.

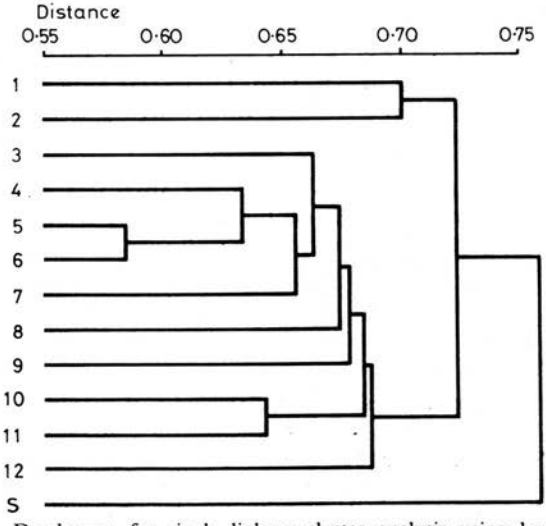
We were gratified to find that all the official selectors clustered better with their colleagues than did the statistician, so they are presumably using their special knowledge in making their judgments. The correlation ($r = 0{\cdot}17$) between the statistician's marks and the mean of the others was significant at the 1 % level, however, suggesting that format and general scientific approach are important factors in selection.

The widespread use of non-standard abbreviations for items of equipment, chemical compounds, drugs, operations, and diseases hindered our appreciation of the abstracts. Explaining the abbreviation the first time it is used does not make the abstract any more readable, though it may make it comprehensible with effort. The banning of all non-standard abbreviations would reduce by a small amount the data that could be presented in an abstract (no bad thing, we reckon) but would more than compensate by making it much more digestible by selector and participant alike.

COULD THE PROGRAMME BE PICKED MORE ECONOMICALLY?

Twelve physicians and surgeons, with an average age in the 40s, read these abstracts. Extrapolation of our own reading time suggests that they devoted at least 200 man-hours to the task in aggregate; this may be an underestimate, since most of the assessors, though fluent in English, had it as a second language. The time of these selectors, who are bound to be prominent members of the profession, is expensive and limited. Any further increase in the number of abstracts could easily overload the system.

We have therefore calculated what difference it would have made to the programme had fewer selectors been employed or had each marked only some of the abstracts. We shall refer to the top 25 and 50 papers picked by the whole 12 selectors as "the best" 25 and 50.



Dendogram for single linkage cluster analysis using 1-r as distance function. Official assessors are indicated by numbers 1-12 and the statistician by S. For explanation see text.

We have compared with them the selection that would have been made by all permutations of six, or of three, selectors. We aggregated the marks of these subgroups to see how their top 25 and 50 compared with the official best 25 and 50.

The most successful (or representative) group of six would have chosen 45 of the best 50, and the most successful three would have chosen 40 of the best 50. The programme could have been picked much more economically, with little injustice, if we could have chosen three selectors with foreknowledge. Inspection of the most successful groupings in our study, however, has not suggested ways in which they could have been picked in advance. They could therefore only be assigned this dubious honour in second and subsequent years, and we doubt whether there would be many volunteers to read all abstracts year after year. Moreover, we do not yet know whether performance is consistent from year to year.

It is therefore of greater interest to know what would have transpired with random selection of assessors. Groups of six selectors would have picked anything from 27 to 45 (mean 38) of the best 50 and an average of 19 of the best 25. Groups of three selectors would have chosen an average of 33 (SD 3·6) of the best 50 and 15 of the best 25.

The method most likely to appeal to selectors is the distribution of each abstract to a random selection of three out of the 12 assessors, so that each has an equal quarter share of the total work. Simulation studies show that this procedure leads to a mean of 32 (SD 2·5) of the best 50 and 14 of the best 25 being picked. This is about the same result as we obtained by asking three selectors to do the whole job but the outcome is a little more predictable, as shown by the smaller standard deviation.

Comment

This last method, which we commend as equitable and practicable, even in the face of an avalanche of abstracts, contains some rough justice. An international congress is expensive. The Helsinki meeting was attended by over 1500 delegates from as far afield as Australia, Japan, and Western America. Even those travelling from Newcastle, using group travel and cheap accommodation, spent £230-330. The selection of a good programme is therefore important and worthy of the effort now expended on it. A well-tried method should not be lightly discarded. Nevertheless, the combined opinions of 12 selectors can only approximate to the choice of all participants.

Most of the "errors" that would have been committed by spreading the load as we suggest represent displacement of papers from just above the cut-off point to just below or vice-versa. When the problem is an embarrassing superfluity of good abstracts this cannot be disastrous. The best 50 abstracts do not necessarily lead to the best 50 papers, and the marking process

is only the first step in constructing a balanced programme. We therefore feel that the modest measure of injustice inherent in our proposed scheme is an acceptable price to pay for speedier and less exhausting work by the selection committee. A late deadline for submitting abstracts and an early announcement of the programme are other benefits that would accrue from spreading the selection load.

We are grateful to the president of EDTA, Professor Jørn Hess Thaysen, and the members of its council for permission to carry out and publish this study and for their helpful comments on the draft manuscript.

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(*Accepted 13 December 1977*)

Printed in Great Britain by Bourne Offset (Iver) Ltd., Iver, Bucks.

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J. biosoc. Sci. (1984) **16**, 291–297

BEDTIMES OF 11 TO 14-YEAR-OLD CHILDREN IN NORTH-EAST ENGLAND

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Summary. As part of a longitudinal study of diet and dental caries, 405 Northumberland children initially aged 11–12 years recorded the time of going to bed on 3 consecutive days on 5 occasions over a 23-month period. The bedtimes were recorded in a personal diary and checked at private interview. Mean bedtime for the 212 females changed from 21 hr 44 min at age 11 years 7 months to 22 hr 11 min at age 13 years 3 months. Bedtime for the 193 males was, on average, 9 min later than that for the females. There was a consistent relation between bedtime and social class, with social class IV + V children going to bed, on average, 14 min later than social class I + II children. When the average age of the children was 13 years 3 months, 13% of them went to bed at or after 23.00 hr, and they were latest to bed on Saturday when their average bedtime was 22 hr 59 min.

Introduction

The time of going to bed is an important nightly event yet, in the United Kingdom, there is little information on the bedtime of children and adolescents. Newson & Newson (1968) reported the bedtime of children aged 1 and 4 years, and showed a strong relationship between bedtime and social class, with the social class I + II children going to bed earlier than children from social classes III, IV and V both at ages 1 year and at 4 years.

In order to provide information for those interested in the social behaviour of young adolescents and to provide baseline information required to assess changes in bedtime over time, data are presented from a survey of over 400 11–14 year old children in north-east England. The children were participating in a longitudinal observational survey of diet and dental caries. Because there was previous evidence that eating sugary foods shortly before bed was particularly harmful to teeth (Palmer, 1971), the children's bedtime was also recorded as part of the dietary assessment.

Materials and method

Four hundred and sixty six children aged 11–12 years, in their 3rd year at seven middle schools in south Northumberland volunteered to take part in a study which had the principal aim of investigating the relationship between a number of dietary factors and dental caries increment (Rugg-Gunn *et al.*, 1983). The 466 children represented 60% of the eligible children in these schools; 405 children completed all aspects of the 2-year study, conducted between September 1979 and July 1981, and it is their data which are reported. Diet was assessed by the completion of a 3-day diet diary followed by a private interview with a dietitian on the 4th day; this was repeated five times over the 2 years for each child. The 3-day diet diaries were issued to the children with full instructions as to how they should be completed. The days were specified so as to achieve a representative sample of all days for each child over the five dietary surveys (15 days total per child). For each day, the time of going to bed was also recorded by the child and checked at interview by the dietitian. Bedtime was the time the children went to bed and did not take into account any reading in bed or the time the lights were actually switched off. No comment on the diary contents nor dietary advice was given at the interview. The social class was obtained for each child (Registrar General, 1970). No information was available on sibship size and parity, or on the menarcheal state of the girls.

When the data were categorized according to the sex or social class of the child, the mean bedtime over the 3 days of each survey was calculated for each child and used as a basis for Table 1 and Fig. 1. When the data were categorized according to

Table 1. Mean bedtimes* recorded by 193 males and 212 female adolescents from north-east England during five surveys between average ages 11 years 5 months and 13 years 3 months by sex and social class

Survey	Age (years: months)	Sex		Social class†		
		M	F	I + II	III	IV + V
1	11:7	21·50	21·44	21·44	21·43	21·55
		(0·36)	(0·38)	(0·39)	(0·34)	(0·41)
2	12:0	21·58	21·55	21·49	21·57	22·02
		(0·49)	(0·49)	(0·52)	(0·45)	(0·56)
3	12:5	22·08	21·51	21·52	21·59	22·05
		(0·40)	(0·40)	(0·38)	(0·41)	(0·41)
4	12:9	22·10	22·00	21·53	22·04	22·13
		(0·44)	(0·43)	(0·50)	(0·43)	(0·41)
5	13:3	22·22	22·11	22·07	22·18	22·22
		(0·42)	(0·37)	(0·37)	(0·41)	(0·40)
N		193	212	85	172	118

* In hr and min to nearest min.

† Excluding very small numbers in social classes VI, VII and VIII.

SDs (between subjects) in parentheses.

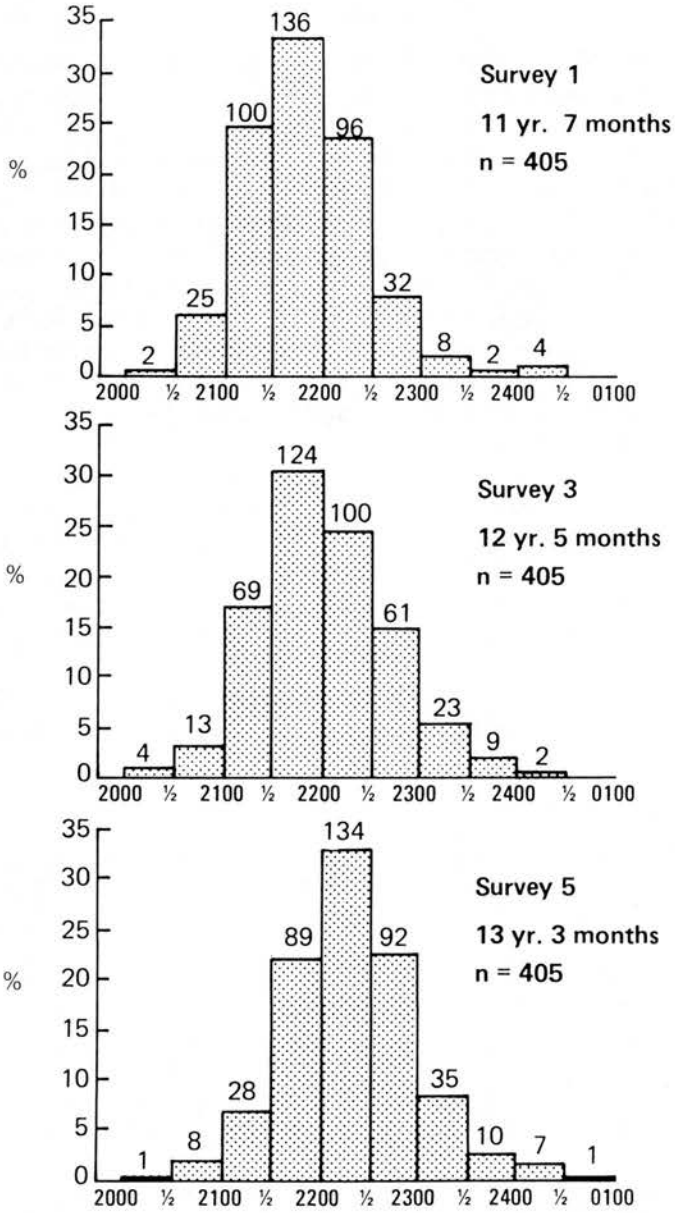

Fig. 1. Frequency distribution of the bedtime (averaged over 3 days) recorded in three surveys, in 405 adolescents, when the subjects had mean ages of 11 years 7 months, 12 years 5 months and 13 years 3 months.

day of the week (Table 2) or month of the year (Fig. 2), each day for each child was treated as independent. Bedtimes are presented as hours and minutes rather than as decimal hours.

Results

The mean age of the 405 children (193 males, 212 females) at each of the five surveys ranged from 11 years 7 months (survey 1) to 13 years 3 months (survey 5) (Table 1). Twenty-one per cent of the children were in social class I + II, 42% in social class III and 29% in social class IV + V; the remaining children (30) have been omitted because of the very small numbers in social classes VI, VII and VIII. At all five surveys, the boys went to bed later than the girls—on average, 9 min later (Table 1). A relationship between bedtime and social class was observed consistently at all five surveys, with the social class IV + V children going to bed, on average, 14 min later than the social class I + II children (Table 1). For both sexes and all three social class groupings, bedtime became progressively later with age. For example, the boys had a mean bedtime of 21.50 at age 11 years 7 months (survey 1), 22.08 at age 12 years 5 months and 22.22 at age 13 years 3 months, a change of 32 min over 20 months.

From the frequency distributions of bedtimes (Fig. 1), it can be seen that 21.30 to 22.00 was the modal bedtime at 11 and 12 years of age, and 22.00 to 22.30 at age 13 years. While 6·7% (27 children) went to bed, on average, before 21.00 at age 11 years (survey 1), 4·2% (17 children) did so at age 12 years (survey 3) and only 2·2% (9 children) at age 13 years (survey 5). The percentage of children going to bed at or after 23.00 rose from 3·5% at survey 1, to 8·4% (survey 3), to 13·0% at survey 5.

The average bedtimes on the 7 days of the week are given in Table 2. Except for survey 1, where bedtime was latest on Thursday and Sunday, the children went to

Table 2. Mean bedtimes* recorded by 405 adolescents during five surveys between ages 11 years 7 months and 13 years 3 months according to day of the week

Survey	Age (years: months)	Day of week						
		Mon	Tues	Wed	Thurs	Fri	Sat	Sun
1	11:7	21·37	21·37	21·39	22·26†	21·46	21·50	22·06
		(0·47)	(0·43)	(0·45)	(1·07)	(1·05)	(0·59)	(1·07)
2	12:0	21·36	21·43	21·49	21·50	22·19	22·37	21·47
		(1·26)	(1·17)	(0·44)	(0·50)	(0·58)	(1.10)	(0·54)
3	12:5	21·46	21·46	21·48	21·50	22·31	22·39	21·49
		(0·43)	(0·38)	(0·44)	(0·43)	(1·02)	(1·05)	(0·45)
4	12:9	21·51	21·49	21·48	21·49	22·34	22·49	21·57
		(0·46)	(0·40)	(0·38)	(0·43)	(1·01)	(1·20)	(0·50)
5	13:3	22·02	22·08	22·05	22·12	22·37	22·59	22·06
		(0·41)	(0·43)	(0·39)	(0·43)	(0·50)	(1·07)	(0·43)

* In hr and min, to nearest min.

† Less than 20 days recorded

SDs (between days) in parentheses.

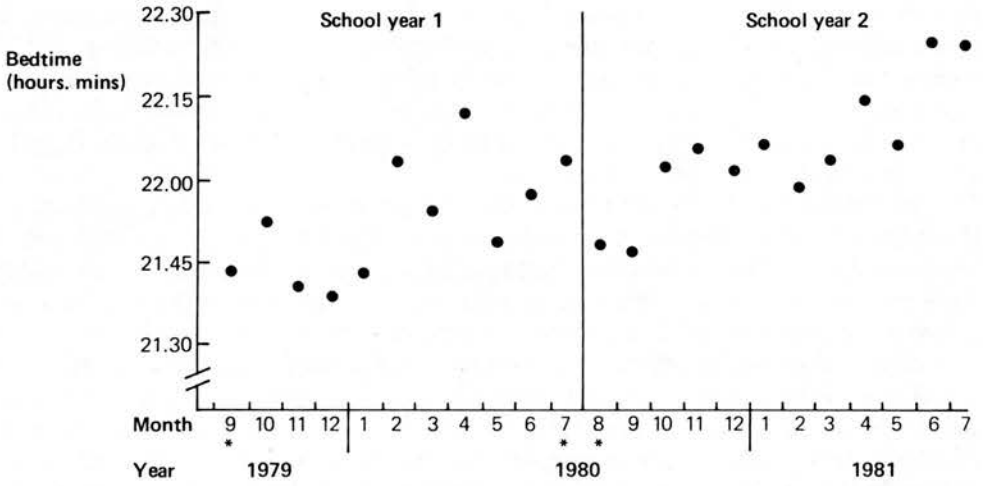

Fig. 2. The mean bedtime (in hr and min) recorded by 405 adolescents (15 days per subject spread over 2 years) according to the month of the year over a 2-year period. *Data based on 50–100 days; data for all other months based on over 100 days.

bed latest on Friday and Saturday nights. The mean bedtime on Saturdays at survey 5 (mean age 13 years 3 months) was 22.59. Variances were large, especially for Saturday.

The mean bedtime recorded during each of the 23 months of the study is shown in Fig. 2, in relation to (a) the months of the year (numbered 1–12) and (b) the school years (from August to July). No clear relationship was observed between the calendar month and bedtime.

Discussion

The main findings from this investigation were that the majority of the children studied, aged 11–14 years, went to bed between 21.30 and 22.30, boys went to bed later than girls, lower social class children went to bed later than social class I + II children and that there was a steady progression of bedtime with age.

Although it was not the prime purpose of the study to record the children's bedtime, the time of going to bed would appear to have been recorded conscientiously. Since the recording of this information was not linked to any enquiry other than dietary intake, it is hoped that the answers were truthful. Although the participation rate was relatively low, the sexes were evenly matched and the social class distribution was similar to that found in this area of south Northumberland. Almost all the data refer to school term-time since it was not possible to interview during holidays. All days of the week, though, were recorded. The days recorded in August refer to the last few days of the school holiday. Very occasionally, bedtimes will have been recorded by children when they were ill, since these diaries were accepted as valid estimates of the child's diet as long as they were

able to attend school on the 4th day for interview. It should be appreciated that the mean bedtime over 3 days was the unit used in compiling Table 1 and Fig. 1. This means that standard deviations (Table 1) refer to inter-subject variation only. Likewise, because intra-subject (between days differences are removed, the ranges of the bedtimes given in Fig. 1 are less than they would have been if all the days for all subjects had been treated independently.

The finding that lower social class children go to bed later than social class I + II children is in agreement with previous data for young children (Newson & Newson, 1968). This is probably because of the special emphasis which middle class parents put upon children being refreshed for school work for the next day (Newson & Newson, 1982, personal communication). A similar social class trend was observed in adults by Palmer, Harrison & Hiorns (1980) who reported that social class IIM (farmers) males in rural Oxfordshire had the longest sleep hours while men from social classes IIIM, IV and V had the shortest sleep duration. Although there was a very strong correlation between social class and school stream in the children in our study, it is not known whether the lower social class children were actually deprived of sleep.

The finding that boys went to bed later than the girls was observed consistently at all ages. The average difference of 9 minutes between the sexes is in close agreement with data reported by Palmer *et al.* (1980) where adult males went to bed 11 minutes later than the females. They also found that the sleep duration was longer in the females than in the males, but it is not possible to compare this variable between surveys, since time of waking was not recorded in our study.

There was a marked tendency for Saturday night to be the late night to bed and this is presumably related to the custom for social functions attended by adolescents or their parents to be held on this night and reduced pressure to get up as early as usual the next day. After the first survey, bedtime on Sunday night was very similar to bedtime on the four nights, Monday to Thursday. In a study of the sleep habits of 21-year-old Australian students, Johns *et al.* (1971) found bedtimes on Friday and Saturday nights to be 1 hr 20 min later than on other nights of the week. However, because of the considerably later time of waking on Saturday and Sunday, they reported that the duration of sleep was greater at weekends than in the week. While Johns *et al.* (1971) reported that the students slept longer in winter than in summer, no clear relationship between month of the year and bedtime was observed in our study.

This would appear to be the first report of the bedtime of young adolescents in the United Kingdom categorized by sex and social class. The findings provide baseline information which should be of importance to educationalists, sociologists and those interested in health care.

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Received 21*st February* 1983

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Society of Physical and Mathematical Biology of the GDR
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European Symposium on Biostatistics / Medical Statistics in Medical Studies and Medicine Related Disciplines as well as in Specialization and Postgraduate Training

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AN MSC COURSE IN MEDICAL STATISTICS

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Summary

The Departments of Statistics and Medical Statistics, and the Health Care Research Unit, in the University of Newcastle upon Tyne run jointly a one-year postgraduate course in Medical Statistics. By providing a mixture of advanced statistical theory and practical experience in analysing real medical data, the course aims to give graduates with a good first degree in statistics the necessary analytical, interpretative and communicative skills to become competent medical statisticians.

Introduction

There has been a Master of Science degree in Statistics in the University of Newcastle upon Tyne for many years. This is awarded to students who have successfully followed an advanced course of lectures and written a dissertation incorporating the results of a practical data-analytic study. Since 1983 there has also been an MSc course in Medical Statistics, a joint venture by three University Departments.

Participating Staff

The organisation of the course is the responsibility of the Department of Medical Statistics, which is in the Faculty of Medicine and consists of a professor, a senior lecturer, two lecturers and one research associate. Also in the Medical Faculty is the Health Care Research Unit, a multidisciplinary team funded by the Department of Health and Social Security. Three lecturers from this Unit -- in Medical Statistics, Medical Data Processing, and Health Economics -- contribute to the course. Of the professor and five lecturers in the Faculty of Science's Department of Statistics, perhaps four will give courses in a particular year. Thus there are about 12 main contributors each year to the lecture courses, giving expertise in a wide range of specialties.

Lecture Courses

In the first two terms (11 weeks and 10 weeks) lecture courses, with practical sessions, are given on Design of Experiments (20h), Design of Clinical Trials (15h), Epidemiology (30h), Exploratory Data Analysis (20h), Generalised Linear Models, including GLIM (55h), Multivariate Analysis (30h), Survival Analysis (20h), Time Series Analysis (20h), and Interactive Statistical Computing (50h).

In the third term students take four short courses (or more if they wish) of 12 hours each, from options on Medical Diagnosis and Decision Theory, Statistical Computing Packages, Analysis of Repeated Measurements, Health Economics, Assessment of Health Care Provision, Simulation of Biological Systems, and Statistical Genetics.

Other Activities

In addition to the lecture courses and their associated practicals, each student has three practical sessions where he gathers data for himself: he makes stereological measurements of the volumes of the nucleus, mitochondria, Golgi apparatus and lysosomes of a liver cell; he counts, using a microscope with an eyepiece graticule, the numbers of red cells in various dilutions of blood; and he carries out a survey on schoolchildren, measuring height and weight and recording age, sex and family size. These practicals give the student a clearer insight than many statisticians have experienced directly of the difficulty (and boredom) of data-collection, and make concrete such concepts as components of variance.

A series of ten seminars by members of staff from other departments in the Faculty of Medicine is a particularly useful and interesting aspect of the course. Typical subjects are 'Problems of Measurement in Experimental Pathology' (a Consultant Pathologist), 'Research in a Clinical Environment' (a Professor of Dermatology), 'Practical Problems in Large-Scale Surveys' (a Professor of Geriatrics), and 'Medical Ethics' (a retired Professor of Medical Physics who is chairman of the District Medical Ethical Committee). Thus the students are made aware of the place of statistics in medical research, and the variety of attitudes towards statistics held by members of the medical profession.

Further contact with medical research workers is established when students join members of staff in consultative sessions in which researchers wishing to design or analyse a study discuss their problems with a medical statistician. Later in the year students may be more directly involved with medical students carrying out research for the Bachelor of Medical Science degree.

Two fairly large analyses are carried out by students over the Christmas and Easter vacations. The first typically involves using GLIM on an unbalanced, partially designed experiment and is chosen because the 'design' imposes difficulties of analysis and interpretation. The second is typically a logistic discriminant analysis of a case-control study where the difficulty of interpretation is caused by high correlation between the variables measured. The final project, which is written up in the form of a dissertation, can be on any topic related to medical statistics or epidemiology, and the data may come from any department in the Medical Faculty or the Newcastle Teaching Hospitals. This project takes 3 months. The student is given guidance, but is expected to communicate directly with the person who has collected the data, and to take responsibility for its analysis and interpretation.

Computing Facilities

The course is based on the analysis of real data; even the most theoretical of the lecture courses make considerable use of computers. Staff and students in the Department of Medical Statistics have access to 5 terminals, 6 Sirius microcomputers, 1 Superbrain microcomputer, 2 matrix printers and a graph plotter. A Clearway network connects the microcomputers, the printers and the plotter, and provides 2 ports to the main University computer (an IBM370/168) to which the terminals are also connected. Students have sole use of 4 of the microcomputers, and share (if necessary) the other facilities. All the microcomputers and three of the terminals support APL, the language principally employed for routine analysis in the Departments of Statistics and Medical Statistics.

Many of the lecture courses are conducted using a microcomputer or a terminal attached to television monitors. Students have access to the microcomputers, and the software which they have used throughout the course, in the written examinations.

Student Assessment

Students are assessed on their performance in carrying out practical assignments during the year in each course, and in particular on their dissertation and on the two analyses they do in the 'vacations'. They are also marked on an oral presentation on one of these projects, and each student has a **viva voce** examination on his dissertation, carried out by two medical statisticians (one not from Newcastle) and a professor of clinical medicine.

There are three written examination papers with a variety of styles of question. There are purely theoretical questions:

Define the **spectrum** of a stationary process.

Suppose that two stationary processes $\{X_t\}$ and $\{Y_t\}$ are related by the set of linear equations

$$Y_t = \sum_{j=-\infty}^{\infty} a_j X_{t-j} \qquad \text{where} \qquad \sum_{j=-\infty}^{\infty} a_j^2 < \infty$$

Derive a relationship between the spectra of $\{X_t\}$ and $\{Y_t\}$.

Suppose that $Y_t = f(t) + U_t$, where $f(.)$ is a smooth, locally quadratic trend and $\{U_t\}$ is a stationary process. Develop an estimator for the spectrum of $\{U_t\}$.

There are practical questions which, because of the availability of microcomputers, may deal with a considerable amount of data or a sophisticated technique. One of the shorter questions is presented here:

In a study of patients with low blood pressure, comparison of 30 hypotensive patients with 20 normotensive patients showed that 30% of the former group died within a certain time as against only 5% of the latter. Construct a 2x2 contingency table for these data and test the significance of blood pressure as a prognostic sign using

(a) a χ^2 test without continuity correction
(b) a χ^2 test with continuity correction
(c) Fisher's exact test
(d) an odds ratio test
(e) a likelihood ratio test.

Discuss and compare the results of these five tests, stating clearly the conclusions you reach.

There are also more discursive questions such as:

> Give an account of the factors which influence the choice between interviews and postal questionnaires. Use examples to illustrate your argument.

and

> Discuss the ways in which medical statistics and the ethics of clinical experimentation affect one another.

Discussion

The Newcastle MSc in Medical Statistics differs in many respects from other MSc degrees in Statistics. It has a large number of staff with varied backgrounds, each teaching subjects in which they have considerable expertise. In particular the participation of medical research workers in data-gathering practicals, seminars, consultations and the examination process is regarded as invaluable. These close links with colleagues ensure that the techniques of analysis which are taught are relevant to the data currently being gathered. This, we hope, ensures that our graduates will have the credibility to be accepted by medical research workers elsewhere. Of prime importance to a medical statistician is his ability to communicate his ideas to his medical colleagues, and this can only be done if he is conversant with their attitudes and their problems, as well as with his own subject. We believe that individuals with a good statistical background can obtain this expertise in our MSc course.