ASPECTS OF RIBONUCLEIC ACID ESTIMATION

by

Betty Mobbs

DEPARTMENT OF ZOOLOGY

UNIVERSITY OF EDINBURGH

Thesis presented for the Degree of Master of Science
in the University of Edinburgh
from
The Department of Zoology, The Ashworth Laboratory
October, 1960.

CONTENTS

ABBREVIATIONS

A	Angstrom unit
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
μμg.	10^{-12} gms.
PCA	Perchloric acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
S	Svedberg unit
SN	Supernatent
SRNA	'Soluble' RNA
U.V.	Ultra-violet

INTRODUCTION.

About twenty years ago, the foundation for the localization and quantitative cytochemical study of ribonucleic acid (RNA) was laid by Caspersson (1936) and Brachet (1942), using the natural absorption of ultra-violet radiation by the nucleic acids and their affinity for basic stains respectively. At the same time, Claude (1943) was devising a biochemical technique for the study of cell fractions isolated by differential centrifugation. The development of these techniques, augmented by the use of radioactive isotopes, has established beyond any doubt the importance of RNA in the biosynthesis of protein. It would be difficult to overestimate the contribution which the use of biochemical methods has made to our knowledge of the role of RNA in the cell, but, as with all techniques, they must be employed with an awareness of their limitations.

The tissues used are not usually homogeneous populations of cells: for instance, in the liver, an organ widely used for the investigation of protein synthesis, although parenchymal cells account for eighty-five per cent of the cytoplasmic volume, only fifty to sixty per cent of the total number of cells are parenchymal, the rest constituting connective tissue, blood cells, blood vessels, macrophages, etc. As Hogeboom and Schneider (1955) point out, even morphologically identical cells may differ in biochemical properties. The techniques used often involve homogenization of the tissue, followed by the separation of various fractions by ultra-centrifugation of the cell-free extract. During these procedures, there is a possibility of morphological and biochemical alteration of the fractions, and redistribution of material

from one fraction to another. There is also the possibility that two or more types of particle have the same sedimentation constant, but different biochemical compositions (Danielli 1953). It is therefore important to make morphological and biochemical checks whenever possible to ensure that these errors are kept to a minimum.

However carefully used, biochemical methods, which deal with amounts of material of the order of 10^{-6}gms. at the lower end of their range, are unable to solve certain problems of cellular metabolism, for instance, those concerned with the growth of the cell between divisions, or with the processes which take place during division or during differentiation. Some of these problems can be dealt with at a biochemical level using homogeneous population of cells, such as a culture of micro-organisms dividing synchronously; but perfect synchrony is difficult to obtain, and the methods by which even partial synchrony can be achieved (e.g. temperature shocks, variations in light intensity) are likely to upset the metabolism of the cell (Scherbaum, 1957a; Iwamura and Myers, 1959). Probably filtration techniques such as that used on Escherichia coli by Maruyama and Yamagita (1956) are more satisfactory, but are not applicable to all types of cell. For the type of problem mentioned above, then, quantitative cytochemical methods capable of dealing with amounts of material found in a single cell (i.e. of the order of 10^{-12}gms.) have been developed.

Cytochemical methods also suffer from some of the sources of error mentioned in connection with biochemical ones, such as the possibility of extraction and redistribution of material during fixation. In addition to these, there may be other sources of inaccuracy, some of which will

be discussed in greater detail later.

Before a quantitative cytochemical technique can be used with confidence, therefore, it is essential to check it against another accepted method, either a cytochemical or a biochemical one. It may be of interest to note some examples of such studies to illustrate this point, mostly in connection with the estimation of deoxyribonucleic acid (DNA) rather than that of RNA.

In 1950, Ris and Mirsky put forward evidence demonstrating that, provided certain conditions are fulfilled, it is permissible to use the intensity of Feulgen staining as a measure of the amount of DNA in the nucleus. They showed this by the use of model systems and by comparing the amounts of stain in erythrocyte and liver nuclei of various vertebrates with values obtained biochemically on a known number of nuclei. Provided the degree of ploidy was taken into account they found good agreement between the biochemical and the cytochemical results.

A similar investigation was carried out by Leuchtenberger et al. (1951) on mammalian liver, spleen, and kidney. These authors also obtained good agreement between the amount of DNA in isolated nuclei as estimated by Caspersson's method of ultra-violet microspectrophotometry and by biochemical methods (Leuchtenberger et al., 1952 a & b).

Ultra-violet absorption methods were used by Walker and Yates (1952 a and b) in a study comparing the DNA values obtained for several types of erythrocytes and sperm with the corresponding values found by other authors using biochemical techniques. Using tissue culture cells which had been filmed when living, Walker and Yates extended

their investigation to include actively growing and dividing cells. They measured the ultra-violet absorption of the nuclei at known points in the life-cycle of the cells and compared the values obtained with the intensity of Feulgen stain in the same nuclei. Both methods showed that there is a gradual increase of material during interphase, but there was a discrepancy in the rates of this increase obtained by the two techniques. Since the Feulgen staining of cells is generally considered specific for DNA, whereas ultra-violet absorption at the wavelength used is specific for certain types of bonds found in all purines and pyrimidines,* the discrepancy between the ultra-violet and the Feulgen data may be accounted for by the presence of DNA precursors in the living nuclei.

Firket (1958) also studied the synthesis of DNA during the growth of tissue culture cells, using Feulgen staining and an autoradiographic method (the incorporation of tritium-labelled thymidine into DNA) and, like Walker and Yates, found that synthesis occurred during interphase.

The investigation of nucleoprotein changes in a bacterially induced plant tumour by Rasch el al. (1959) demonstrates how cytochemical and biochemical methods may complement each other. Estimated biochemically, RNA values showed scarcely significant differences between the stems forming tumours and the control stems, whereas the cytochemical method used (cytophotometry after Azure B staining) made obvious localized increases in RNA content involving comparatively few cells. These increases had been masked by the inclusion of large numbers of normal cells for the biochemical analysis. Conversely,

---

* as well as for certain other substances e.g. ascorbic acid

during the first few days of tumour growth, the large increase in DNA content found by biochemical methods was absent when DNA was measured by the Feulgen technique. The authors suggest that the 'extra' DNA may represent a labile component which is lost from the cells during the cytochemical procedures, or that it is distributed uniformly throughout the host tissue and that therefore the increase per cell is too slight to be detected photometrically, or that contamination has occurred during the biochemical procedure. The use of an autoradiographic method would perhaps help to elucidate this point.

An example of the checking of one cytochemical method of measurement against another is given by Mendelsohn and Richards (1958), who measured the intensity of gallocyanin-chrome alum stain in the same ascites tumour cells by scanning microphotometry and by the two wavelength method. They obtained a high degree of proportionality in the results.

The importance of using living cells whenever possible is illustrated by the work of King (1959), who compared the nucleic acid and protein content of the cytoplasm of ascites tumour cells determined by ultra-violet absorption methods with the dry mass obtained by interference microscopy. He found good agreement between the two determinations in fresh cells, but the results from fixed cells were more variable.

Some of the above investigations were facilitated by the fact that, with certain reservations, e.g. in the case of aneuploid or polyploid cells, all the non-dividing somatic cells of an animal contain virtually the same amount of DNA, the diploid amount, while

normal mature sperm contain the haploid amount of DNA. Such cells as nucleated erythrocytes and sperm can therefore be used as standards in the comparison of techniques and to check reliability. In the case of RNA, however, there are no such standards. Even in cultures of micro-organisms and protozoa, there seems to be quite a large biological variation between the individual cells at any particular stage in the life-cycle (Brachet, 1957; Mitchison and Walker, 1959). This is an important reason for the scarcity of cytochemical methods for the quantitative investigation of RNA compared with the number of methods for its detection. It is also more unstable than DNA and exists in several different fractions within the cell, which may require different techniques for their investigation.

The first part of this dissertation consists of a brief review of the cytochemical methods available for the estimation of RNA, together with results I have obtained using some of these methods, and the conclusions to be drawn from them. The second part is a description of the organization of RNA within the cell, with reference to the cytochemical techniques which have been used for its investigation, followed by the record of an attempt to devise a technique at a biochemical level which could be adapted for use on a cytochemical level for the extraction of one RNA fraction from intact cells.

Most of the literature referred to was published after 1950: reference to earlier work may be found in the reviews mentioned.

PART I. CYTOCHEMICAL METHODS FOR RNA ESTIMATION.

1. MICROSPECTROPHOTOMETRY.

The most widely used methods of estimating the amount of RNA in single cells or in parts of cells are based on the measurement of the absorption of radiation of a particular wavelength i.e., they are microphotometric methods. The first of these takes advantage of the fact that nucleic acids and proteins have a high natural absorption in the ultra-violet range of wavelength 230-280 mμ, while the second group relies on the staining of RNA with basiphilic dyes. Owing to the difficulty mentioned in the introduction - that of the absence of satisfactory standards of reference for RNA measurements at a cytochemical level, and to doubts about the specificity of the stains and their conformity to the absorption laws, results obtained by staining procedures are not generally regarded with the same confidence as those obtained with ultra-violet techniques. Ultra-violet microspectrophotometry also has the advantage of being one of the few cytochemical techniques which can be used on living cells, although its usefulness in this respect is limited partly by movement of or within living cells, but chiefly by the fact that irradiation above a certain limit with ultra-violet light is damaging to living matter. Davies (1950) and Walker and Davies (1950) found that under the conditions of their experiments, it was possible to take only two or three photographs of living chick fibroblasts without the cells being affected, although up to fifteen exposures could be given before the damage became obvious.

The basic instrumentation for both types of microspectrophotometry is the same; namely:-

i) a suitable source of radiation

ii) a system for selecting radiation of the desired wavelength(s), either a monochromator or a filter, and for directing it through

iii) a microscope. In the case of ultra-violet microspectrophotometry it is of course necessary to use quartz or reflecting optics (Wilkins, 1953) owing to the opacity of glass to ultra-violet radiation.

iv) a system for the measurement of the amount of radiation transmitted. This may be done photo-electrically or photographically.

Complex instrumentation has been developed to increase the accuracy and rapidity with which measurements can be made: for complete discussions of this aspect of microspectrophotometry see Caspersson, (1947, 1950, 1955, 1956, 1957), Caspersson et al. (1957), Davies and Walker (1953), King and Roe (1953), Nurnberger (1955), Pollister and Ornstein (1959), Swift (1956), and Walker (1956, 1958).

Photometric analysis is based on two laws:

i) Lambert's Law $A_\lambda = k_\lambda l$,

ii) Beer's Law $A_\lambda = k_\lambda c$,

where A_λ = absorbance (extinction) of monochromatic radiation of wavelength λ

k_λ = absorptivity (extinction coefficient) = $\frac{\epsilon}{M}$

where ϵ = absorbance of one cm. cuvette length of molar solution

M = molecular weight

l = cuvette length in cms.

c = concentration in gms. per litre.

As $A_\lambda = \log_{10} \frac{I_o}{I}$

where I_o = intensity of incident radiation of wavelength λ

I = intensity of transmitted radiation of wavelength λ

$$\log_{10} \frac{I_0}{I} = kcl \quad \text{(Lambert-Beer Law)}$$

The conditions under which these laws apply are comparatively easy to satisfy on a macroscale, where one is dealing with a homogeneous solution of known cuvette length, and it is easy to test an unknown substance for conformity with the Beer-Lambert law by using several known concentrations and cuvette lengths. When dealing with biological material the situation is more complex. The possibility of measuring the absorption of the small amounts found in cells means that they occur in very much higher concentration than those used in macroseale photometry, and it is possible that the Beer - Lambert law therefore does not apply in the cell, although it may do so at the concentrations used on the macroscale. It is difficult to measure the 'cuvette length' of a cell, and this may vary from point to point. There is also the possibility that the absorption curve and the distribution of the absorbing substance may be influenced by the cellular environment, by changes in pH, by interactions with other molecules and by the effects of fixation, irradiation, and extraction procedures (Attardi, 1957; Beavan et al., 1955; Caspersson, 1950; Davies, 1954; Davies and Walker, 1953; Swift, 1955; Walker 1956, 1957;). It is possible to test the validity of the absorption laws in cells to some extent:for instance, by measuring the absorption of sections of varying but known thickness (Lambert's Law), and by measuring the absorption of cells containing the substance in question in the same amounts but at different concentrations (Beer's Law).

Other possible sources of error are introduced into microphotometric procedures of the form of the specimen and by the distribution of the chromophores in it. These are fully discussed in the reviews already mentioned, and will be dealt with only briefly here.

Errors due to non-parallel light and the form of the specimen.

If one considers non-parallel light passing through an absorbing object, it is clear that the outer rays of the illuminating cone have to pass through a greater amount of absorbing material than do the inner rays, giving rise to a spuriously high absorption value. The size of the error depends on the refractive index of the object and on the numerical aperture of the objective, and is smallest for an object of low refractive index and a small numerical aperture.

The volume of an object is generally calculated as a cylinder whose cross-section is measured at the object plane (the so-called 'projected area'). With a thick object and non-parallel light, it is obvious that this calculated volume is smaller than the actual volume of absorbing material through which the light is passing. Errors due to chromophores being out of focus are also likely to arise in thick objects.

Distribution errors.

As was mentioned above, the Beer-Lambert law applies to homogeneous solutions in which all the light not transmitted by the object is absorbed by it. This is not always the case in biological specimens, in which absorbing substances are often far from homogeneously distributed. This gives rise to 3 possible sources of

error.

(a) An amount of absorbing material distributed heterogeneously, for example in particles or clumps, will give different extinction value from the same amount distributed homogeneously. The size of the error introduced will depend on the ratio of the areas of the chromophores and the background, and can be minimised by using a very small measuring spot. (Also see p. 13).

(b) Unspecific light loss. An erroneously high absorption value may result from:

(i) reflection of light from boundaries separating areas of different refractive indices - this is independent of wavelength: and (ii) scatter, which is also caused by heterogenity of refractive index, but in this case increases with decreasing wavelength. For particles of a size approximately one-tenth of the wavelength of light used, scatter may be corrected for by Rayleigh's law

$$\text{Loss of light} \propto \lambda^{-4}$$

but for biological objects in the ultra-violet, the scattering particles are larger than this, and the correction applied varies from (loss at λ)^{0} to (loss at λ)$^{-4}$, where λ is about 312mμ, a wavelength at which the specific absorption due to nucleic acids and protein is very low.

These unspecific light loss errors are always lowest in living material, and in fixed material may be reduced by mounting the specimen in a medium of similar refractive index to itself. Caspersson (1950) decreased sharp refractive index differences at boundaries within the specimen by mounting frozen-dried material in

water-free glycerin, and other workers have used glycerin containing zinc chloride (Rudkin and Corlette, 1957) and butyl methacrylate polymerized with ultra-violet radiation (Swift, 1955). Many mounting media have their own drawbacks (Walker, 1958) and there is also the point, at least in unstained material, that the more nearly the refractive indices of the specimen and the mounting medium are matched the more difficult it is to find and focus the specimen.

(c) Orientation of molecules. Commoner (1949) drew attention to the possibility of obtaining too low an extinction value owing to orientation of the chromophorés. In the same case of complete orientation, the extinction value could not rise above 0.3.Molecular orientation can be detected by birefringence and does not seem likely to cause serious errors except in cells where the concentration of nucleic acids is very great, such as in sperm or Nissl substance, but the possibility that it may be increased by such procedures as fixation should be borne in mind: this applies to all distributional error.

Whenever possible, it is desirable to measure the total extinction of a cell or organelle in order to compare it with the corresponding part of other cells, rather than to compare measurements made at a few points only, since the volume (and therefore the concentration) of cells may vary widely, especially under different physiological conditions. For example, Gordon and Nurnberger(1955) found that in liver,conditions of stress such as fasting or brief exposure to cold produced a substantial increase in protein and DNA concentration in the cells without significantly changing the amounts

present. In order to calculate the total extinction, it is necessary to measure the extinction at a large number of points (each point being very small - about $1\mu^2$ - to minimise the distributional error) and multiply the mean of these values by the area of the specimen. This is tedious and time-consuming, and various scanning devices have been devised to facilitate measurements and increase their accuracy (Caspersson et al, 1957; Deeley, 1955; Deeley et al 1954). The extinctions of all the spots measured may be integrated automatically so that only one reading is necessary for the measurement of the total extinction of the trace across the specimen (in the case of line scanning) or of the whole specimen (in the case of area scanning). An alternative method is provided by photographic recording, after which the negative is scanned together with the negative of a density wedge of known extinction, so that the film density can be translated into terms of extinction of the specimen. Photoelectric recording is generally considered to be more accurate than the photographic method, but the latter does have some advantages (Walker and Davies 1950): it forms a permanent record both of the extinction and of the morphology of the object, and makes it easy to relate the two; and there is no uncertainty as to whether or not the specimen was in focus at the time of measurement.

Whether scanning procedures or point measurements are used, it is always preferable in microspectrophotometry to deal with whole cells isolated by microdissection or in smears rather than with sections, which introduce additional possibilities or error in calculating the volume of the object.

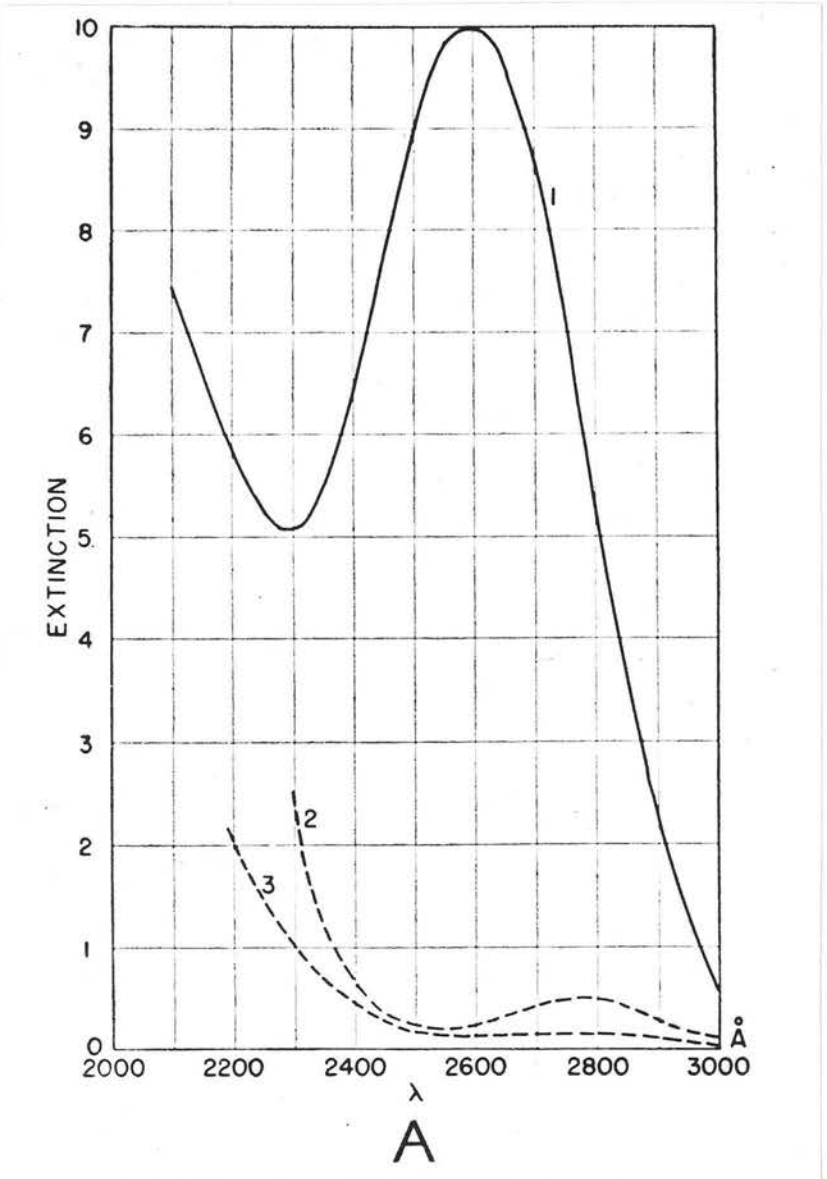


Fig.1. Absorption curves of nucleic acid and protein in similar concentrations. (1) DNA, 1cm. and 0.5% (2)serum albumin, 0.5% (3) protamine sulphate, 5% From Caspersson,(1950).

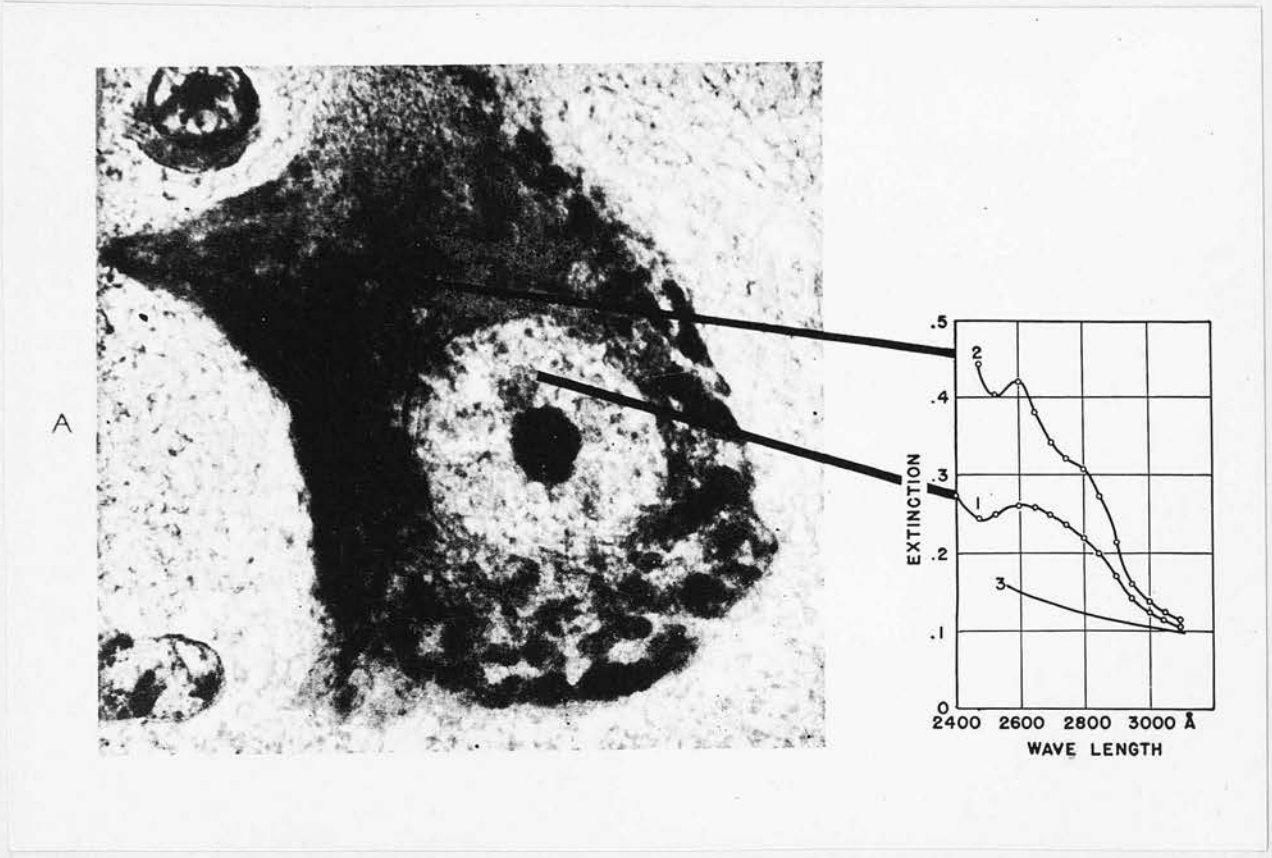


Fig.2. Anterior horn cell with absorption spectra of points in the cytoplasm and nucleus. From Caspersson (1950).

The above comments apply equally well to microphotometry in ultra-violet and in visible light : further aspects of the two methods will be examined separately.

A. Ultra-violet microspectrophotometry.

(i) 'in situ' in the cell.

The use of U.V. radiation for microscopy permits high resolution of cellular detail, partly owing to its short wavelength, and partly to the high absorption of ultra-violet light by certain parts of the cell. Apart from a few exceptions,* light in the range of wavelengths 230-290 mμ is specifically absorbed by the pyrimidine rings in nuleic acid components (with a peak at about 260mμ) and by certain amino-acids (tyrosine, tryptophane, and to a lesser extent phenylalanine) found in proteins (with a peak at about 280mμ) (Fig. 1). In 1936, Caspersson devised the technique of ultra-violet microspectrophotometry, which uses these properties for the investigation of the composition and amounts of nucleic acids and proteins in individual cells or parts of cells. (Fig. 2). The sensitivity of this method is such that it is possible to measure 5×10^{-14}gms. of nucleic acid present in an area $1\mu^2$ with an accuracy of ten per cent (Walker, 1956). At 260mμ the absorption of protein is very small compared to that of the same concentration of nucleic acid (See Fig. 1) : Caspersson (1950) gives a figure of one-twentyfifth, Moberger (1954) of one-fortieth.

Many ultra-violet microspectrophotometric studies have been carried out on the nucleus and its components as these are highly absorbing and can be easily defined. As far as the quantitative study of RNA is concerned, Caspersson and his co-workers have used

---

* this refers to substances found in cells

this method to investigate RNA-protein relationships in several types of protein-synthesising systems, such as gland and haemopoietic cells, tumour cells (also see Moberger (1954) and micro-organisms. This work is summarised in Caspersson's monograph (1950). Because of their large size and high RNA concentration, nerve cells are well suited to investigation with this this type of technique and have been extensively studied. (Attardi, 1953, 1957; Nurnberger, Engstrom and Lindstrom, 1952; Gordon and Nurnberger, 1955; also see p. 30).

The investigation described below is similar to one carried out by Mitchison and Walker (1959), in which they measured the relative amounts of RNA in individual cells of the fission yeast Schizosaccharomyces pombe at different stages in the cell cycle and thus derived a curve for the pattern of RNA synthesis between divisions. The present experiments differ from those of Mitchison and Walker in that the absolute amounts of RNA have been calculated in order to compare the results with biochemical estimations on the same material, and in that one set of experimental conditions were such that the results could be compared with those obtained with another cytochemical technique. The effect of using aqueous extraction techniques on the cells was also examined.

Material

The fission yeast Schizosaccharomyces pombe (N.Y.C.Y. 132) is very well suited to cytological investigation, and already a good deal of information has accumulated on the growth of populations and of individual cells under various conditions (Mitchison, 1957; Harnden, 1957; Faed, 1959). Except for slight surface irregularities

due to division scars, the cells are cylindrical with rounded ends. The diameter (c.3.5μ) remains constant throughout the life cycle of the cell. The cell's regular shape makes cytological measurements easier than, for instance, does the irregular shape of tissue culture cells. Under optimum conditions (log phase culture in 2% W/V Oxoid wort broth at 33°C) the length increases from approx. 6μ after cleavage to approx. 18μ about three quarters of the way through the life cycle (the mean generation time at this temperature is 2 hours (Smith, (unpublished)), when the constant volume stage is reached (Mitchison, 1957; Mitchison and Walker, 1959). During this stage, the central vacuole, which probably represents, or at least contains, the nucleus, divides, and this is followed by the growth of the cell plate across the centre of the cell. When growth of the cell plate is complete, cleavage occurs by division of the cell into two (usually equally sized) daughter cells and the rounding of the two new ends. Mitchison (1957) has shown that the increase in dry mass and volume (except during the constant volume stage) is linear throughout the life-cycle of the cell, so that the stage in interphase which the cell has reached may be estimated from its length. Faed (1959) carried out similar measurements of cell volume, but used a technique in which the experimental conditions were kept constant, and in most cases found that the volume-time curve was positive at least at the beginning and end of the period of volume increase.

S. pombe is also well suited to RNA estimation. The concentration of RNA is relatively high, and the DNA:RNA ratio has been found to be low (P.D.Mitchell, in press). For the purpose of ultra-violet

microspectrophotometry this amount of DNA can be ignored.

Method.

10 ml. stock cultures containing approximately 25 x 10^6 cells per ml. 2% Oxoid wort broth i.e. cultures in the stationary phase of growth, were maintained in McCartney bottles at room temperature. All experiments were carried out on cultures in the logarithmic phase of growth. These were obtained by adding 1 ml. of stationary culture to 9 mls. sterile distilled water and shaking thoroughly; 0.1 ml. of this suspension i.e. 25 x 10^3 cells of the original culture were inoculated into 10 mls. sterile broth. After 17 hrs. incubation at 33°C., the culture contained 3-6 x 10^6 cells per ml. The cells were then spun down at 1500g, and treated in one of six ways.

(a) Unfixed and untreated cells.

The supernatent medium was decanted from the cells, which were resuspended in 1 ml. distilled water. An equal quantity of 20% gelatin heated to about 35°C was mixed with the suspension, some of which was smeared with a glass rod on a 1 mm. thick quartz slide and covered with a 0.1 mm. coverslip, which was pressed down well.

(b) Frozen-substituted cells.

9 mls. of the supernatent medium was decanted, and the cells were resuspended in the remainder. Some of the cells were smeared on quartz coverslips and were fixed by quenching in liquid propane cooled to -140°C by immersion in an outer container of liquid nitrogen. The coverslips were then rapidly transferred to absolute methanol cooled to -75°C with a mixture of dry ice and ethanol. The methanol was allowed to warm up to 4°C overnight, and to room temperature the

next day. The cell smears were air-dried and stored over calcium chloride in a dessiccator at 4°C until needed. They were then transferred to chloroform, and were finally mounted on a quartz slide in Merck's liquid paraffin.

(c) Unfixed and RNase extracted.

The medium was decanted from the pellet of cells, which was extracted with 2% PCA for 20 min. at 4°C to remove the acid-soluble nucleotides (Oger and Rosen, 1950). The PCA was then washed off with distilled water and the cells were resuspended in 10 mls. of a solution of ribonuclease (Worthington) at a concentration of 0.4 mgm. per ml. 0.2 M ammonium acetate-0.2 M ammonium carbonate buffer of pH 7.6, and incubated at room temperature for one hour. After centrifugation and removal of the enzyme solution, the cells were washed twice with the buffer. They were then resuspended in ml. of buffer, mixed with gelatin and mounted as in (a).

(d) Frozen-substituted and RNase extracted.

After preparation and fixation as in (b), air-dried cell smears were re-hydrated and extracted in the same way as the unfixed cells. After extraction they were dehydrated, transferred to chloroform and mounted in liquid paraffin.

(e) Unfixed and PCA extracted.

The medium was decanted from the pellet of cells, which were resuspended in 5 mls. of 10% PCA at 4°C and were left overnight at this temperature for 18 hours. (Ogur and Rosen, 1950). The cells were spun down and the supernatent was discarded. The cells were washed twice with disilled water, resuspended, and mounted in gelatin as in (a).

(f) Frozen-substituted and PCA extracted.

Frozen-substituted and air-dried cell smears were re-hydrated and extracted in the same way as the unfixed ones in (e). After extraction, they were dehydrated, transferred to chloroform, and mounted in paraffin.

The light source used for microspectrophotometry was a high-pressure mercury arc. The monochromator was designed by Seeds and Wilkins: matt black baffles were inserted at appropriate points to minimise the effect of stray light within the monochromator. The latter, and the microscope used (Beck, Model No. 4196) are described by Walker (1956). The microscope was fitted with two reflecting objectives, one being used as a condenser, and a Beck 35 mm. camera. The cells were focussed in visible light and photographed on Kodak Microfile Pan film at 312 mμ and 265 mμ with exposures of 1 and 3 secs. respectively. It was found that slight adjustment was needed to obtain a well-focussed photograph in the ultra-violet. The specimen was removed from the microscope stage and a density-wedge of rhodium steps on quartz manufactured and calibrated by Hilger and Co. was placed in front of the film and photographed at 312 mμ and 265 mμ. The film was then developed in D19b for 5 min. at 20°C. Film densities were measured with a recording microdenstometer (Walker, 1955); an aperture limited the measuring spot to an area corresponding to 1μ diameter on the cell. One tracewas recorded for each cell along its length, and the areas beneath each curve were determined with a planimeter. This area was divided by the length of the base of the trace, which corresponded to the length of the cell, the resulting value

representing the average height of the curve. The calibration curve obtained from the trace of the density wedge was used to content this value to A_λ. Cell areas were measured by projecting their images on to squared paper calibrated by the projection of the image of a micrometer slide photographed at the same magnification. The absolute amount of RNA per cell was then calculated from the formula:

$$m = \frac{A_\lambda a}{k}$$

where m = mass RNA in gms.

A_λ = absorbance at wavelength λ

a = area in $10^3 cm.^2$

k = absorptivity of RNA at wavelength λ

Reference RNA.

The absorptivity (k) of a sample of RNA depends on three factors - the nucleotide composition of the sample, the pH at which the absorbance is measured, and the degree of polymerization of the sample.

(a) Nucleotide composition.

The absorptivities at 265 mμ of the four main nucleotide components of RNA* have been calculated from data taken from Beavan et al. (1955), and are set out in TABLE I.

---

*Some RNAS contain very small quantities of other nucleotides (Littlefield & Dunn, 1958 a & b). These are ignored here.

TABLE 1

Nucleotide	265×10^{-3}	M.W.	k_{265} ($= \frac{\epsilon}{M.W.}$)
Adenylic acid	14.0	347.2	40.4
Cytidylic "	8.5	323.0	25.5
Guanylic "	10.3	363.2	28.4
Uridylic "	10.0	324.2	31.0

The value of k for polymerized RNA will depend partly on the proportions in which the four nucleotides occur. The method by which the base ratio of the RNA of S. pombe was investigated is described in the Appendix; the four nucleotides were found to occur in the following ratio:

Adenylic acid	10.0
Cytidylic "	6.5
Guanylic "	9.5
Uridylic "	12.0
	38.0

Assuming for the moment that the absorptivity of RNA is equal to the sum of the absorptivities of its component nucleotides, k_{265} for S. pombe RNA will be:

$$\frac{10 \times 40.4}{38} + \frac{6.5 \times 25.5}{38} + \frac{9.5 \times 28.4}{38} + \frac{12.0 \times 31.0}{38}$$

$$= 31.9$$

(b) pH

The precise form of the absorption spectrum of RNA and its components depends on the pH at which it is measured. The extent of the variation depends on the substance concerned : for instance, it is very large for cytidylic acid, but relatively small for guanylic acid. The k_{265} estimated above is for a mixture of the four main nucleotides in neutral solution in the same proportions as is found in the RNA of s.pombe.

(c) The degree of polymerization.

It has been found that the absorbance of polymerized RNA is always less than that calculated from the sum of the absorbances of its components. The 'hyperchromicity' which occurs on hydrolysis is a phenomenon associated with high-molecular weight polynucleotides, since the absorbances of residue containing up to twenty nucleotides are normal (Beavan et al., 1955) and so are those of the small polynucleotides obtained by ribonuclease digestion. (Schmidt, 1955). Reports of the magnitude of the hyperchromic effect range from 20 - 37% (Beavan et al., 1955; Magasanik, 1955; Schmidt, 1955; Walker, 1956) and it is thought to be due to an alteration of the resonance states of the bases when they are bound in high molecular weight polynucleotides. Magasanik (1955) found that the absorption of two samples of yeast RNA increased by 24% and 37% respectively after alkaline hydrolysis i.e., that the absorbance of the polymerized RNA was 19% and 27% less than the sums of the absorbances of the constituent nucleotides.

Assuming that this effect in S. pombe results in 23% less absorption in the polymerized RNA than would be expected from the constituent nucleotides

$$k_{265} = 31.9 - \left(\frac{23}{100} \times 31.9\right)$$

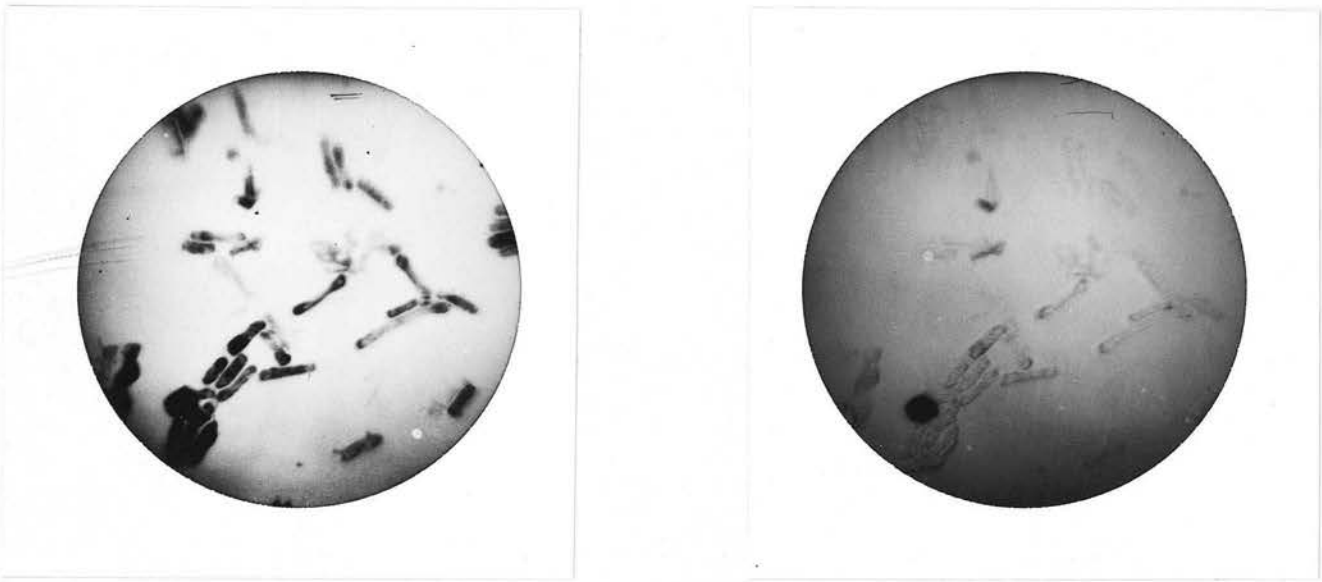
$$= 24.6$$

This is the value for k_{265} which has been used below in calculating the amounts of RNA per cell from the ultra-violet microspectrophotometric data.

(a) (b)

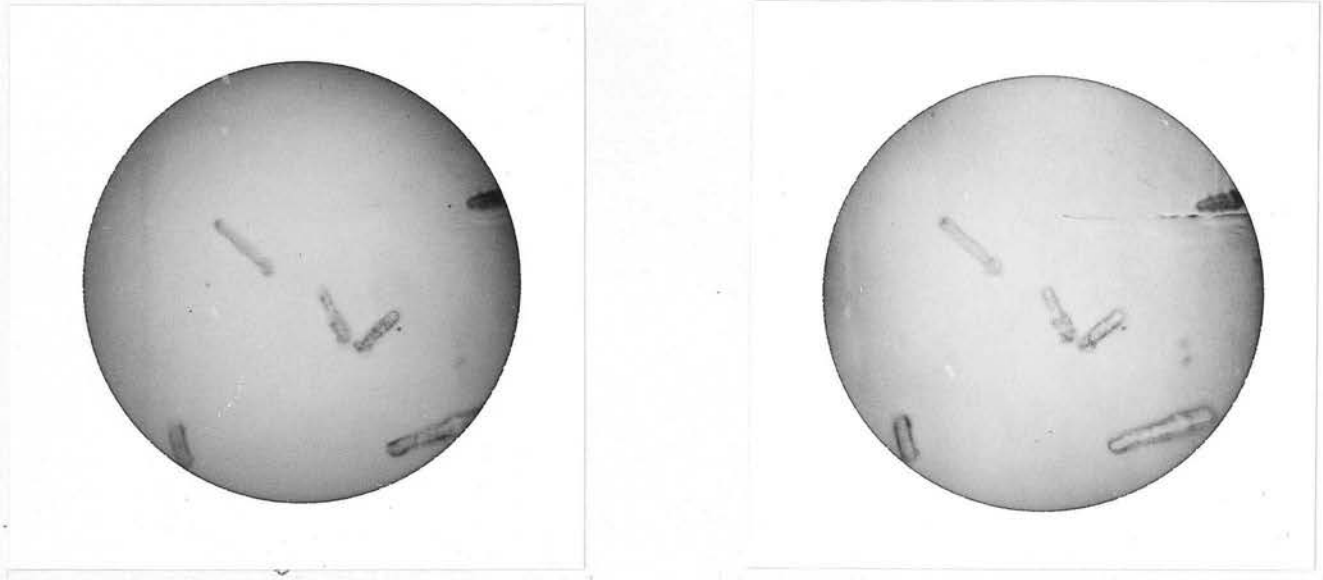
Fig.3. Unfixed and untreated cells of S.pombe mounted in 10% gelatin and photographed at (a) 265 mμ, (b) 312 mμ

10μ



(a) (b)

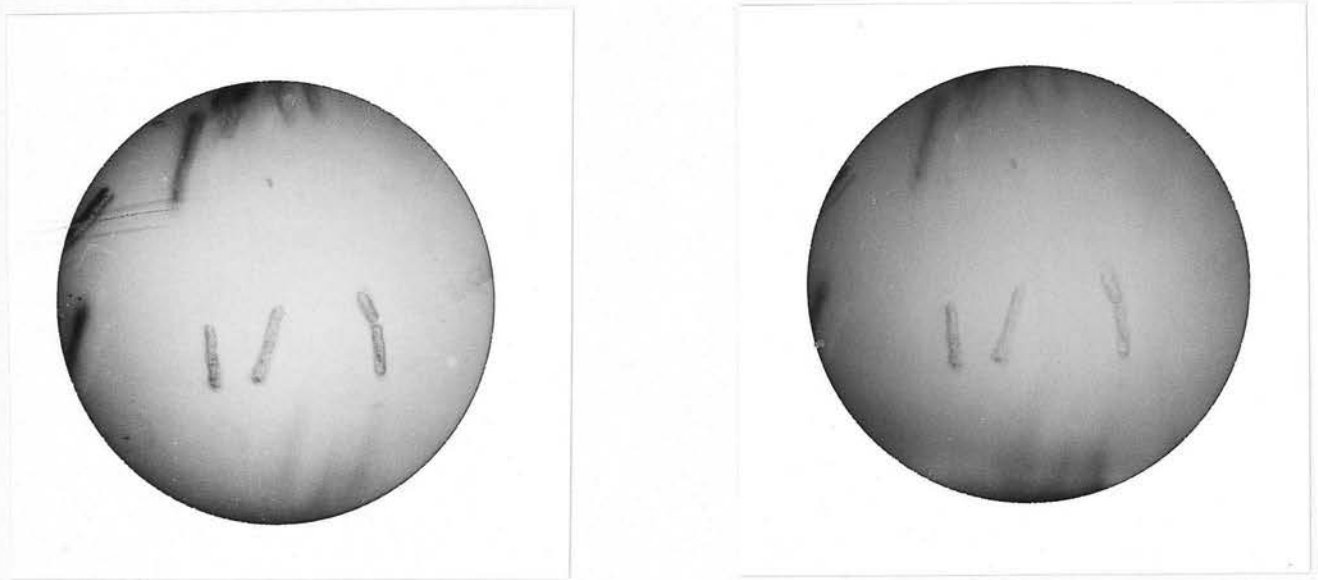
Fig.4. Frozen-substituted cells of S.pombe mounted in liquid paraffin and photographed at (a) 265 mμ, (b) 312 mμ



(a) (b)

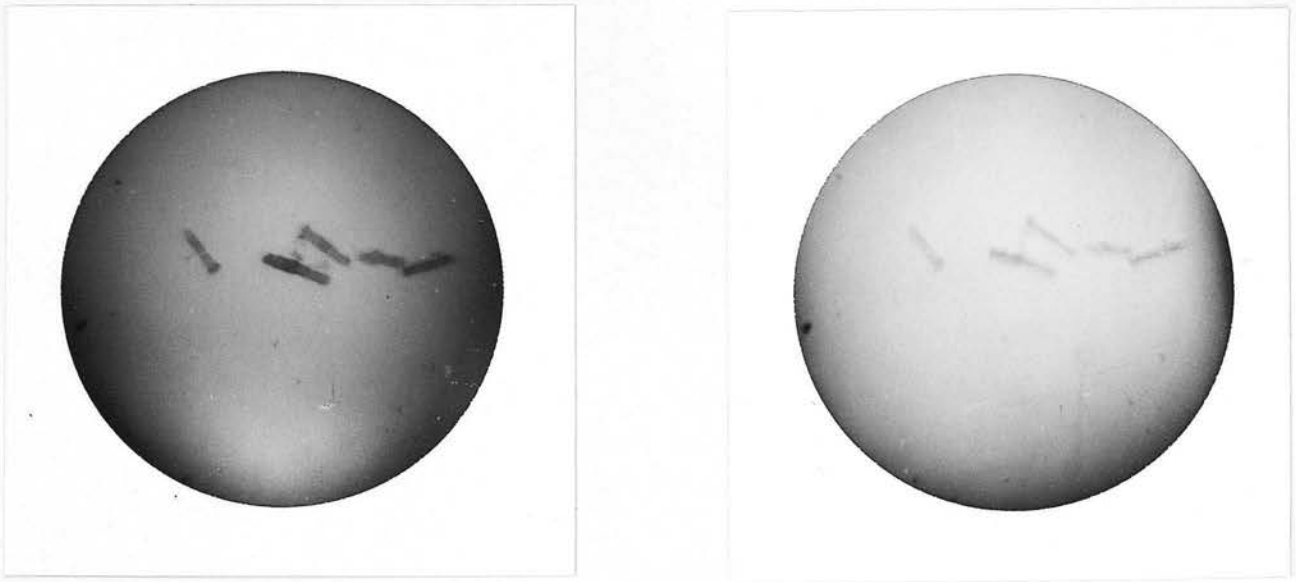
Fig.5. Unfixed cells of S.pombe after extraction with 2% PCA and RNase, mounted in gelatin and photographed at (a) 265 mμ, (b)312 mμ

10μ



(a) (b)

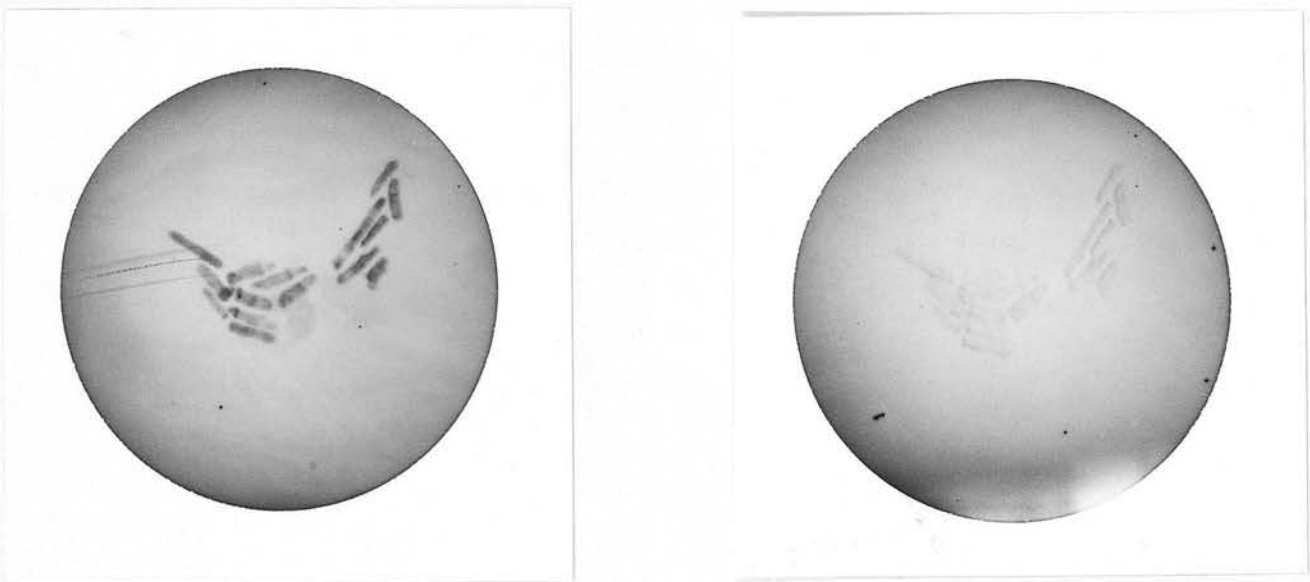
Fig.6. Unfixed cells of S.pombe after extraction with 10% PCA, mounted in gelatin and photographed at (a) 265 mμ,(b) 312 mμ



(a) (b)

Fig.7. Frozen-substituted cells of S.pombe after extraction with 2% PCA and RNase, mounted in liquid paraffin and photographed at (a) 265mμ, (b)312 mμ

10μ



(a) (b)

Fig.8. Frozen-substituted cells of S.pombe after extraction with 10% PCA, mounted in liquid paraffin and photographed at (a) 265 mμ, (b) 312 mμ

Results and Discussion.

(a) Unfixed and Untreated Cells.

The photographs at 312 mμ showed that unspecific absorption and light loss due to scatter was negligible in these cells (Fig.3). The absorption at 265 mμ was due to the presence of polymerized RNA and acid-soluble nucleotides*, so that it is necessary to apply a correction for the latter before the former can be estimated. Mitchell (in press) found that the ratio of RNA to acid soluble nucleotides in exponentially growing cultures was 17:5, so that the acid-soluble nucleotides would account for $\frac{1}{3.4}$ of the total absorption of the cell if the absorption coefficients of polymerized RNA and the nucleotides were the same. Owing to the hyperchromic effect, however, the absorption of the RNA is relatively smaller than that of the acid-soluble nucleotides, the ratio of their respective absorptivities being $17 - (\frac{23}{100} \times 17) : 5$ i.e. the acid-soluble nucleotides account for $\frac{1}{2.62}$ of the total absorption of the cell. This is the correction applied in calculating the results presented in Table 2. In so doing it has been assumed, firstly, that the composition of the acid-soluble nucleotides mixture is the same as that of the polymerized RNA, and secondly, that the proportion of nucleotides to polymerized RNA remains the same throughout the life-cycle of the cell, although Mitchison and Walker (1959) found some evidence of a slight increase in the proportion of nucleotides present before cleavage. The cells have been divided into a convenient number of groups according to their projected area and the mean of the amounts of RNA in each group

---

* The small amount of DNA present has been ignored.

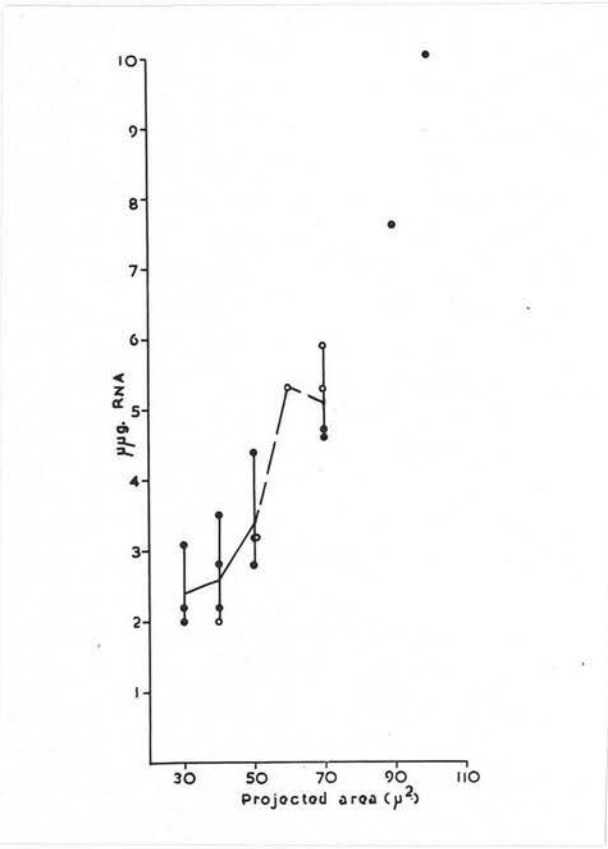


Fig.9. RNA content of unfixed and untreated cells of S. pombe plotted against the projected area. Open circles denote cleaving cells, or cells with cell plates. Points derived from one or two values only are connected by broken lines.

has been plotted against area in Fig. 9.

That there is a good deal of biological variability among the cells of the culture is shown by the variation in the size at which the cell plates appear. Occasional giant cells occur, which contain proportionately large amounts of RNA. If these are ignored, the results show that the amount of RNA per cell is approximately doubled during the doubling of the projected area (which is proportional to volume), increasing from approximately 2.5 µµg. in the smallest cells to about 5.0µµg. in the largest. Mitchell (personal communication) found by biochemical methods that the RNA content of cultures of S. pombe in the exponential phase of growth accounted for 10% of the dry weight of the cells. Taking the value of approximately 55µµg. for the dry weight of a cell at cleavage obtained by Mitchison from interferometric measurements, this means that the RNA content of a cell at cleavage is approximately 5.5µµg., which agrees very well with the value obtained here by the U.V. microspectrophotometric technique. Walker and Richards (1958) state that 5 µµg. in an area of $100\mu^2$ is about the smallest amount of RNA which can be measured with an accuracy of 10% using U.V. microabsorption techniques. The cells used here fall just within this limit, so that any procedure which lowers the amount of RNA present such as some fixation or extraction procedures, would also lower the accuracy of the technique.

The presence of the biological variation mentioned bars any but a tentative analysis of the shape of the synthesis curve. Ignoring cells of exceptional size, and taking into consideration results obtained by another method (see section on Azure B staining of S. pombe), there seems to be a general tendency for the rate of synthesis to

increase during most of the life-cycle, and to fall off during the final quarter of it. This falling off is in accordance with finding of Mitchison and Walker (1959). It was not felt here that the number of data justified the constuction of a synthesis curve by the histogram method of Walker (1954).

Other attempts to investigate the pattern of RNA synthesis between cell divisions have been carried out largely on groups of cells synchronized in various ways, some of which are likely to upset the normal metabolism of the cell. These are summarized by Mitchison and Walker (1959). Prescott (1960) has recently carried out a study of the synthesis of DNA, RNA and protein using small groups of Tetrahymena synchronized by the selection of dividing cells with a pipette. Synthesis of cellular components was measured by the incorporation of isotopically labelled precursors. Prescott found that the synthesis of protein (which can be equated with that of dry mass) follows a linear course throughout the life-cycle. As has been mentioned before, Mitchison (1957) found that the dry mass of S.pombe, measured interferometrically, was also linear between divisions. As regards RNA, the rate of synthesis in Tetrahymena shows a continuous increase throughout the life-cycle, the increase being particularly marked in the second half of the cycle. Owing to the slight loss in synchrony towards the end of the cycle, Prescott is unable to tell whether or not there is a loss in the rate of synthesis just before division, but if this does occur, it is not likely to occupy such a high proportion of interphase time as it appears to in S. pombe.

Since it is established that RNA performs a major role in the synthesis of protein, it might be expected that the rate of protein

synthesis in the cell would parallel that of RNA, but this does not appear to be so, either in S. pombe or in Tetrahymena : in both organisms the rate of production of RNA throughout the life-cycle tends to increase during most of interphase, while that of protein remains constant. This may be because the RNA alone is not capable of synthesising the complete protein molecule, but must be present in the form of ribonucleoprotein to do so. In this case, the amount of protein synthesised at any particular time in the cell-cycle would be a function of the amount of protein available for binding to RNA, rather than the amount of RNA present. In the case of both S. pombe and of Tetrahymena, the linear form of the dry mass and protein synthesis curves could be accounted for if a constant amount (but decreasing proportion) of the protein synthesised were available for RNP formation, the remaining protein being used for structural and metabolic purposes other than that of further synthesis. The rate of RNA formation might then be unrelated to the rate of a protein formation. If this were so, in cells where the rate of RNA synthesis increases during the life-cycle, one would expect the occurrence of free RNA in increasing amounts, the proportion of free RNA: RNP depending on the difference between the rates of RNA and protein synthesis. Results of experiments on micro-organisms in which protein formation, but not RNA synthesis, has been suppressed by chloramphenicol, suggest that RNA formed in the absence of protein is non-functional, and that it exists in the form of an unstable polymer which may nevertheless be present in the form of RNP particles. (Aronson and Speigelman, 1958; Dagley and Sykes, 1960). Aronson and

Speigelman found that these particles have the same sedimentation constant as the normal RNP particles and consider that the unstable polymer is an intermediate stage in the formation of normal RNA, since it is converted into a stable form after the removal of the chloramphenicol and the addition of amino-acids. If the supply of amino-acids in a cell were limited, therefore, it might happen that not all the RNA could be converted into the stable, functional form, available for the synthesis of protein. This may be an additional factor determining the difference in form of the protein synthesis and the RNA synthesis curves in Tetrahymena, and to a lesser extent in S. pombe.

It might be possible to find out how much of the RNP is functional in vivo by carrying out experiments with labelled amino-acids and estimating the proportion of RNP which is carrying the label after the appropriate incubation time.

(b) Frozen-substituted and untreated cells.

In this case, it has been assumed that all the acid-soluble nucleotides have been removed by fixation. In view of experiments to be described in Part II, this assumption seems justified. The non-specific light loss was negligible (Fig. 4), so that no correction was necessary before the amount of RNA was calculated.

The results are presented in Table 3 and Figure 10. It is clear that this method of fixation, followed by subsequent treatment with chloroform results in severe shrinkage of the cells. The latter was accompanied by a slight increase in optical density, but this was not sufficient to compensate for the shrinkage, so that the total optical density for each cell was low in comparison with the unfixed cells.

TABLE 3

Frozen-substituted and untreated cells.

Cell No.	Av. Optical Density (O.D.)	Area μ^2 ($= a \times 10^{-11}$)	Total O.D.	Amount RNA µµg. ($= m \times 10^{-12}$)
1	0.28	14.9	4.2	1.7
2	0.28	14.0	3.9	1.6
3	0.35	15.7	5.5	2.2
4	0.28	18.2	5.1	2.1
5	0.37	16.6	6.1	2.5
6	0.32	13.2	4.2	1.7
7	0.29	14.9	4.3	1.7
8	0.23	10.8	2.5	1.0
9	0.31	13.2	4.1	1.7
10	0.12	21.5	2.6	1.1
11	0.35	15.7	5.5	2.2
12	0.40	17.4	7.0	2.8
13	0.38	14.9	5.7	2.3
14	0.35	17.4	6.1	2.5
15	0.34	9.8	3.3	1.3
16	0.54	33.1	17.9	7.3
17	0.41	17.4	7.1	2.9
18	0.41	16.6	6.8	2.8
19	0.42	20.7	8.7	3.5
20 C. P.	0.32	20.7	6.9	2.8
21 C. P.	0.63	27.3	17.2	7.0
22	0.38	17.4	6.6	2.7
23	0.40	16.6	6.6	2.7
24	0.39	12.4	4.8	2.0
25	0.37	13.2	4.9	2.0

C.P. Denotes the presence of a cell plate.

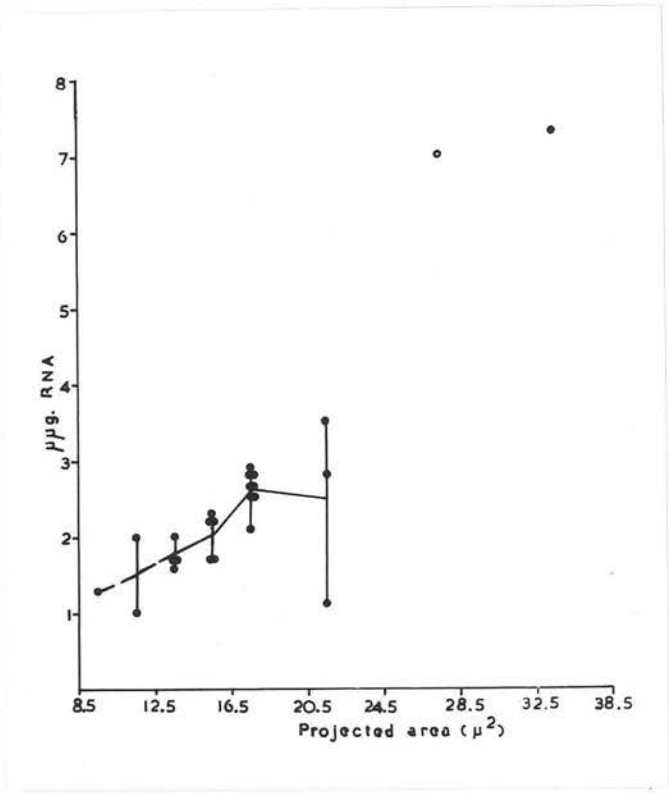


Fig.10. RNA content of frozen-substituted cells of S.pombe plotted against the projected area.Open circles denote cleaving cells, or cells with cell plates. Points derived from one or two values only are connected by broken lines.

The RNA synthesis curve is of the same general shape as for the unfixed cells, and there is an approximate doubling of the amount of RNA during the life-cycle, but the amount is reduced to little more than half the value found in unfixed cells. Davies (1954) has shown that freeze-substitution alone has no effect on a film of egg-white and RNS beyond slightly increasing the non-specific light loss. One can only conclude that in S. pombe freeze-substitution removes part of the RNA; this would also decrease the accuracy of the measurements, for the reason mentioned above. It is known (Sylvén, 1952) that fixation with acetic alcohol or absolute alcohol results in the loss of a considerable amount of material absorbing at 260mμ into the fixation fluid, but Bell (1958) considers that this loss is limited to low molecular weight polynucleotides. It has also been shown that a similar loss occurs during freeze-substitution (Bell, 1959), and it may be that in this case the material extracted includes some RNA as well as relatively low molecular weight nucleotides, and that the loss is increased by the subsequent passage through chloroform, which may increase the permeability of the cell-wall. In the case of S.pombe the discrepancy between the amounts of RNA present in the unfixed and in the frozen-substituted cells increases with the increase in volume of the cells, and it may not be too fanciful to suggest that the RNA extracted represents the RNA referred to above which is non-functional in protein synthesis.

These results emphasise the need for quantitative investigation of the effects of fixation and subsequent procedures used in cytochemical work.

(c), (d), (e), and (f). (Fig.5, 6, 7, 8).

It was found that neither of the extraction techniques used completely removed the RNA from the cells, whether they had been fixed or not. Although no absorption measurements were made on these cells, the negatives showed that the RNase extraction technique increased the unspecific absorption at 312 mμ; this is one reason why, if such a technique is used, it is better to measure the absorption of the material extracted from the cell rather than that of the material remaining within the cell. The cells had undergone a good deal of shrinkage compared to the unfixed and untreated ones, but the unfixed and extracted ones did not shrink as much as those which had been frozen-substituted and extracted. It was concluded from this that freeze-substitution caused a certain amount of shrinkage and that passage through chloroform before mounting in liquid paraffin increased this.

(ii) Ultra-Violet microspectrophotometry of cell extracts.

A method of ultra-violet microspectrophotometry which eliminates many of the difficulties of dealing with inhomogeneous biological material has been devised by Edstrom, and has been used in several studies on the determination of the amounts of RNA in individual nerve cells (Brattgard et al, 1957, 1958; Edstrom and Eichner, 1958; Edstrom and Pigon, 1958a & b; Hyden, 1959) and by Pigon and Edstrom (1959) for the quantitative study of RNA in the ciliate Urostyla. Originally, the method involved the extraction of RNA with RNase, the hydrolysis products being placed on strips of treated cellophane (Edstrom, 1953), or on treated cellulose fibres about 20 μ in diameter. The hydrolysis products can be concentrated on to a

length of fibre of about 40μ and photographed in ultraviolet light together with a density wedge. The amount of RNA can then be estimated as 'in situ' ultraviolet microspectrophotometry. By applying a large potential (6000v) across the ends of the fibre it is possible to separate the individual hydrolysis products electrophoretically and thus calculate the amounts of each from an individual cell (Edstrom. 1956). Edstrom claims an accuracy of ten percent for amounts of RNA in the range of 100-1000μμg., which is about the amount found in neurones. The method has been criticised by Gordon and Nurnberger (1955) on the grounds that in neurones a large part of the cell mass is in processes which are likely to be lost during the preliminary isolation of the cell by microdissection. On the other hand, Brattgard and Hydén (1954), in a paper summarising the work of Hyden and his co-workers, compare the values for RNA content of motor root cells obtained by conventional 'in situ' microspectrophotometry and by Edstrom's microchemical determination, and find the latter give a higher and more accurate result. For large, easily isolated cells, this method has much to recommend it.

Edstrom (1958 a & b) has modified the method to deal with quantities of RNA as small as 10 - 20 μμg., which is nearer the amount per cell found in most of the mammalian tissues which have been investigated (Leslie, 1955). The modification consists of evaporating the extracted RNA hydrolysis products on to a quartz slide, covering them with liquid paraffin and redissolving them in a small droplet of glycerin containing a buffer. This droplet is then photographed in ultraviolet light and the amount of RNA

estimated as usual. This is the method used in the investigation described below, which was undertaken in an attempt to investigate the variation in amounts of RNA during interphase in a population of mammalian cells actively growing and dividing in uniform conditions.

Material

The cells were interphase fibroblasts grown in tissue culture from 17-day mouse embryo heart explants. A thin film of serum exuded from a clot formed from equal volumes of 11-day chick embryo extract and cockerel plasma was spread between the arms of a horse-shoe shaped clot formed by mixing equally sized drops of the same embryo extract and plasma. The explants (about 1 mm^3 in volume) were placed on the film of serum. In this way, the growing cells were nourished by substances diffusing from the horse-shoe into the serum, and it was possible to wash off the serum before fixation so that there was nothing to interfere with the extraction technique. The cultures were grown over well slides at 37°C. After 48 hours, when growth was active, the coverslips were removed and the cultures were washed for about ten minutes with warm Tyrode solution. Excess Tyrode was removed with Kleenex tissue and the cultures were fixed by freeze-substitution. In case any acid-soluble nucleotides remained, the cultures were hydrated and extracted with 2%/PCA at 4°C for 20 minutes. They were then dehydrated, transferred via chloroform to Merck's liquid paraffin and stored at 4°C until they were used.

Method

For extraction, the cover-slip was placed, culture down, over a groove 25 mm. wide and 3 mm. deep in a slide 7 cm. long, 4 cm. wide

and 5 mm. deep. The chamber thus formed was filled with liquid paraffin. Subsequent procedures were carried out on the stage of a phase contrast microscope, using microneedles and micropipettes manoeuvred with a de Fonbrune micromanipulater. The cell selected for extraction was isolated by scraping away surrounding cells with a flat-ended glass rod about 10μ in diameter made by breaking the tip from a microneedle made with a de Fonbrune microforge (de Fonbrune, 1949). Extraction was carried out at room temperature with a solution of 0.4 mgm, per ml. of 0.2 M. ammonium-acetate 0.2 M. ammonium carbonate buffer of pH 7.6. Greenstein et al. (1947) found that for complete digestion of RNA with RNase it is necessary for an electrolyte to be present, and those used here are volatile, so that they do not interfere at later stages of the procedure, Edstrom (1953) has shown that this extraction technique resulted in the complete removal of RNA from the material he used (Carnoy-fixed motor neurones). A drop of the enzyme solution just sufficiently large to cover the cell was placed over it with a micro-pipette about 5μ* in diameter at the tip, and was left for 20 minutes. The droplet was then removed to a blank part of the cover-slip near the edge. This process was repeated twice, so that in all the cell was extracted for an hour. The three droplets were drawn into a fresh pipette of the same diameter, which already contained paraffin to prevent evaporation of the hydrolysate, and the grooved slide and cover-slip were removed from the microscope stage. Another grooved slide covered with a quartz slide 1 mm. thick and without paraffin, was placed on the stage. The micro-pipette

---

* Made by the drop-weight method of De Fonbrune

containing the hydrolysate was arranged so that its tip was just touching the under surface of the slide; the hydrolysate then flowed on to the slide, and at the same time, as the amount leaving the pipette was so small, the buffer began to evaporate, leaving the hydrolysis products together with a little RNase dry on the slide. This process was continued until all the hydrolysate had evaporated and paraffin began to flow from the pipette. The evaporation could be hastened by a small radiant heater fixed to the arm of the microscope, but this had to be adjusted very carefully as the pipette became blocked if evaporation was too quick. The chamber formed by the groove and the quartz slide was filled with paraffin, and the hydrolysate was then dissolved in a droplet of 1 part glycerol: 2 parts buffer from another pipette. The purpose of the glycerol is to raise the refractive index of the droplet so that it approximates that of the paraffin, and refraction effects are minimised. Ideally the diameter of this droplet should not be larger than that of the original cell, but this was not easy to achieve. The quartz slide was then removed from the grooved one, and after excess paraffin had been removed, a quartz coverslip (thickness 0.1 mm.), was placed over the part containing the drop-let, which was then ready for photography in ultraviolet light.

The apparatus used was the same as that described in the previous section, except for the monochromator, which was manufactured by Hilger and Co., and is described by Walker and Davies (1950). The droplets were photographed at 265 mμ only; being a homogeneous solution, there was no need to correct for scatter by photographing

them at 312 mμ. Control droplets containing an equivalent amount of RNase only showed no absorption in the ultraviolet so that it was unnecessary to correct for the absorption of RNase or buffer.

Reference RNA

In the absence of date on mouse fibroblast RNA, it would seem reasonable to take the absorptivity of mouse ascites tumour cell RNA (King, 1959), as the nearest approximation, i.e. $k_{257m\mu} = 18.5$. It is assumed here that RNase digestion gives the maximum hyperchromic effect and that the latter results in a 30% increase in absorptivity. It is also assumed that the A_{257}: A_{265} ratio is 1.1 . This gives a value for k_{265} for digested RNA of 21.8 .

Results and Discussion

Densitometry was carried out as described in the previous section, but as the droplets were large compared to the yeast cells, five traces were recorded for each droplet. The traces emphasised the difference in homogeneity between the yeast cells and the fibroblast extracts.

As before,

$$m = \frac{Aa}{k}$$

Where m = mass in gms.

A = absorbance at 265 mμ.

a = area in $10^3 cm^2$

k = 21.8

The results are set out in Table 4.

TABLE 4

Droplet No.	Av.O.D	Area ($a \times 10^{-11}$)	A x area Total O.D. $\times 10^{-11}$	Amount RNA µµg ($a \times 10^{-12}$)
1	0.045	1092	49.1	20.6
2	0.05	3440	172.0	68.0
3	0.055	1148	63.1	26.6
4	0.09	520	46.8	19.7
5	0.11	1016	111.8	47.0
6	0.05	3380	169.0	71.0
7	0.095	1088	103.4	43.4
8	0.045	1797	81.0	34.0
9	0.05	1513	75.7	31.8

All the cells extracted were growing in the same culture and were near the edge of the outgrowth where growth and division were most active, and where the cells were farthest apart and so comparatively easy to isolate. There should therefore be very little difference in the chemical composition of the cells due to culture conditions.

In view of the results obtained by 'in situ' U.V. microspectrophotometry on S. pombe after freeze-substitution, it is possible that the absorbing material does not represent all the RNA of the cells, part of which may have been removed during fixation. The following remarks therefore apply only to the RNA remaining after freeze-substitution.

As the mean extinctions of the droplets are so low, no great accuracy is claimed for the results, but, (apart from cells 2 and 6) it is clear that the highest value for the amount of RNA per cell (47 µµg.) is very approximately twice that of the lowest value (19.7 µµg.) as

would be expected if doubling of RNA occurred during interphase. It will be noted that the areas of the droplets corresponding to cells 2 and 6 are very large in comparison to the others, which would be expected to result in very low absorbances: the measured absorbances may therefore be inaccurate, accounting for the high RNA values. However, it is possible the association of large droplets areas with a large amount of RNA in the corresponding cells is merely coincidental, and that the RNA increases to more than twice its initial value during interphase. This is supported by data obtained by Newton and Wildy (1959) from biochemical estimations of nucleic acids in cultures of HeLa cells, which are comparable to the mouse fibroblasts used here in that they are mammalian cells which are growing and dividing rapidly. The HeLa cell cultures had been induced by a low temperature shock to divide 'parasynchronously' i.e., although perfect synchrony was not established, there were bursts of division at intervals of 18 hours, the majority of cells dividing within half and hour of each other. Parasynchrony was maintained for at least two divisions after one temperature shock. From the data which Newton and Wildy give, it is possible to calculate the mean amount of RNA per cell at different stages of the cell cycle. It appears that just before division, that while the DNA remained constant at 26µµg. per cell, the total nucleic acid content dropped from 83 to 67 µµg., which would be accounted for by a decrease in RNA from 57 to 41 µµg. At division, this amount would presumably be halved, i.e., a cell which had just undergone division would contain 20.5 µµg. of RNA. It is possible that in the mouse fibroblasts used here, cells 2 and 6 are very late in interphase

and are about to lose RNA prior to division. Use of a film technique such as that used by Walker and Yates (1952) in their study of DNA synthesises in chick fibroblasts would elucidate this point.

B. Microspectrophotometry in visible light.

Staining with basic dyes has been used extensively for the detection and localisation of nucleic acids: indeed, the use of this method by Brachet (1942) provided independent confirmation of Caspersson's findings; that RNA is invariably present in large amounts in cells which are actively synthesising protein. However, the use of basic dyes for the quantitative determination of RNA presents even more uncertainties than did the use of ultra-violet microspectrophotometry. As well as the possible sources of error discussed above common to all microphotometric procedures, the behaviour of the dye itself introduces additional factors. These will now be considered.

1. Specificity.

The binding of basic dyes is thought to be due to the formation of electrovalent linkages by the basic group of the dye and the acid group of the material stained - in the case of the nucleic acids, the free phosphoric acid groups. That this is so is supported by the fact that other cations, such as La^{+++}, and basic proteins, such as protamines and histones, can block the staining reaction by competing for the binding sites on the nucleic acids (Swift, 1955). Factors increasing the number of available phosphoric acid groups, such a rise in temperature, may increase the amount of dye bound (Flax and Himes, 1952). Besides the nucleic acids, substances in the cell which contain acid groupings include proteins with carboxyl groups and muco-

polysaccharides, presumably because they contain sulphonic acid esters (Swift, 1955). When staining for nucleic acids therefore, it is important to stain controls treated with the appropriate enzyme (RNase or DNase) to check for unspecific dye-binding by such substances and for dye absorption on to cell surfaces etc. Staining should be carried out above pH2, as the phosphoryl groups of the nucleic acids are charged above this pH, and below pH5, as carboxyl binding by most basic dyes is then negligible (Swift, 1955). After staining, unbound dye is removed by rinsing in absolute ethanol, or in a higher alcohol, if the dye concerned is soluble in the former. This differentiation in itself introduces a new set of conditions; it may alter the pH, for instance; and this may upset the relationship between dye and substrate.

2. Stoichiometry.

If a dye is to be used for quantitative determination of its substate, the amount bound must be proportional to the amount of substrate present. This is the case with certain dyes in solution with polymerized nucleic acids; deploymerization may increase the amount of dye bound in some cases e.g. with pyronin Y, decrease it in others e.g. with methyl green, and has no effect with e.g. methylene blue. It is extremely difficult to demonstrate a stoichiometric relationship between dye and substrate <u>in situ</u> in the cell unless an independent method is available to confirm this. Ris and Mirsky (1950) established that the intensity of Feulgen staining was proportional to the amount of DNA present in several types of cell by comparing the microphotometric data with those obtained by biochemical means. This was made possible by the constancy of the amount of DNA

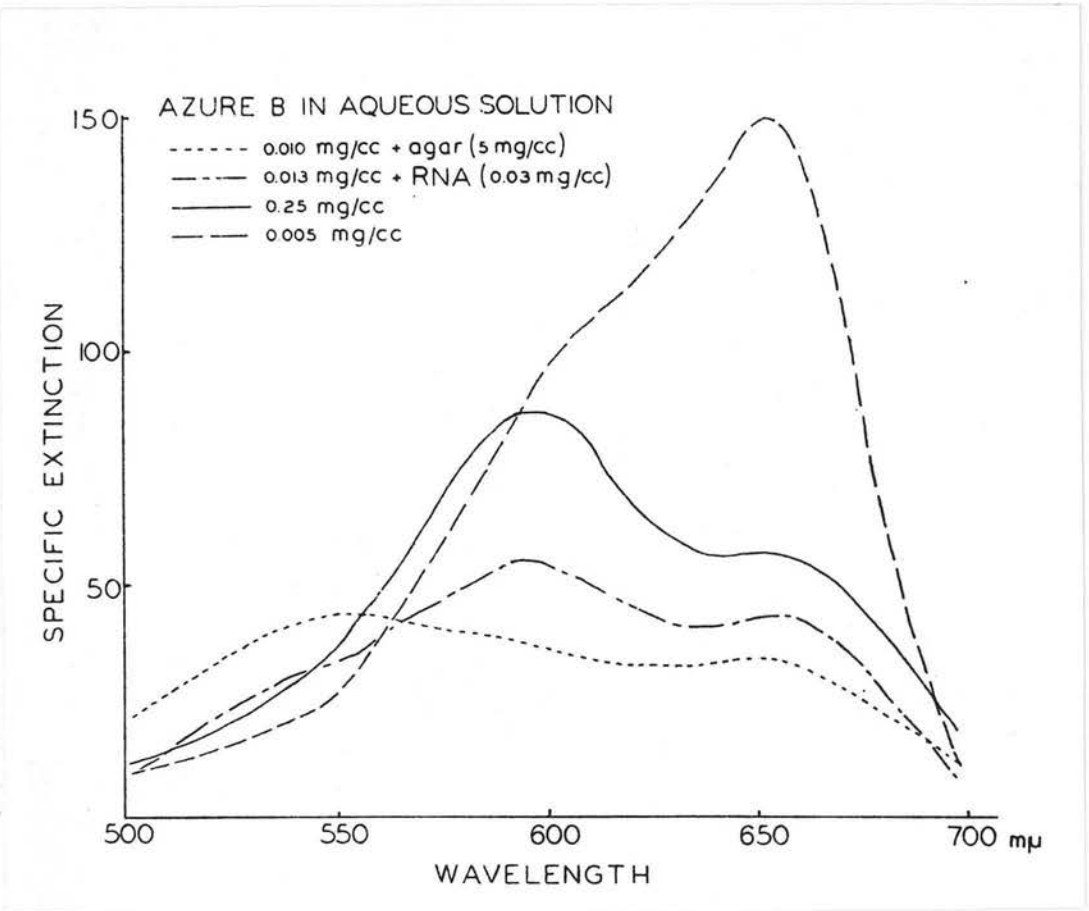


Fig.11. Effect of changing concentration and substrate on absorption curves of Azure B in solution. From Flax and Himes (1952).

in most cells of the same species. But as mentioned before, in the case of RNA no such reference values are available, and it is necessary to check the stoichiometry of the dye by other cytochemical means.

3. Beer's Law and Metachromasy.

Even if stoichiometry betwen dye and subtrate is established, it does not necessarily follow that the light absorbed by the dye is proportional to its concentration i.e. that Beer's law is obeyed. Even in solution, some dyes exhibit metachromasy; as the concentration is increased, the absorption characteristics change, so that new peaks appear on the absorption curve and the dye colour changes (Fig.11). This is thought to be due to the polymerization of the dye molecules by Van der Waal's forces to form dimers and higher polymers (Michaelis, 1947; Swift, 1955; Swift and Rasch, 1956, Sylvén, 1954). Besides its dependence on the concentration of the dye, the degree of metachromasy may vary with the nature of the substrate, the pH, and the temperature (Flax and Himes, 1952).

It is clear that dye binding by the nucleic acids is a very complex matter: the amount bound depends on an equilibrium governed by many factors, such as by the amount and nature of protein present, some of which will be bound to nucleic acids, probably blocking some groups which would otherwise accommodate dye molecules; by the pH within the cell; by the temperature; by the ability of the dye molecules to interact with each other, and by the effects of fixation on the material.

The sources of error due to the geometry of the specimen and to inhomogeneous distribution of the chromophores described earlier apply here, but some additional remarks are appropriate. Possible random error due to fluctuations in the photometric apparatus can be minimised by making several measurements on the specimen. This cannot be done in ultraviolet light as it may damage the cell. Owing to the longer wavelength of visible light, unspecific light loss due to scatter is not likely to play such an important part as in the ultraviolet range. Distributional errors, on the other hand, are important, and have been minimised by various means.

i) Fixation.

Fixation with formalin after treatment with thirty per cent sucrose has been found to give homogeneous distribution of nucleoprotein in the cell (Ris and Mirsky, 1950; Moses, 1952), but it seems that such treatment has disadvantages which may outweigh this. Swift (1955) mentions that formalin combines with amino groups, thus altering the balance of electrostatic charges on nucleoprotein complexes, and Davies (1954) found that forty per cent of the cytoplasmic matter as measured as by ~~interference~~ ultraviolet microscopy was lost after formalin fixation. It also caused shrinkage and increased unspecific light loss at 313mμ.

ii) Crushing condenser.

Davies, Wilkins and Boddy (1954) devised a condenser with a hemispherical top lens which can be raised to crush appropriately mounted cells, flattening them and redistributing the absorbing material in a more homogeneous way. Besides minimising errors due to

inhomogeneity, the crushing condenser also reduces those due to thick objects, and lowers the extinction of dense objects to a level which can be measured accurately (A = 0.2-0.7).

iii)The two-wavelength method.

Ornstein (1952) and Patau (1952) independently devised a method which enables accurate microphotometric measurements to be made on inhomogeneous objects. It is known as the two-wavelength method, and involves the measurement of the absorption of the specimen at wavelengths λ_1 and λ_2, where $A_{\lambda 1} = 2A_{\lambda 2}$ in a comparable but homogeneous, specimen. The ratios of the absorption of the inhomogeneous object at the chosen wavelengths depends on the degree of inhomogeneity, and by substituting the ratio in the appropriate formula, the true absorption at λ_1 can be calculated.

iv) Scanning methods.

As in ultraviolet microspectrophotometry, the technique of scanning the specimen with a very small aperture so that errors due to inhomogeneity are almost eliminated has been developed. Such an instrument was designed by Deeley(1955) and has been used mainly for the measurement of DNA stained by the Feulgen technique (Atkins et al., 1959; Atkin & Richards, 1956; Richards & Atkin, 1959; Richards et al, 1956). If this instrument is used with the crushing condenser described above, out of focus errors are also reduced, as are those due to very dense or thick objects.

Mendelsohn and Richards,(1958)measured the absorption of the same nucleic stained with gallocyanin-chromalum by both the two-wavelength method and with the instrument designed by Deeley, and found almost

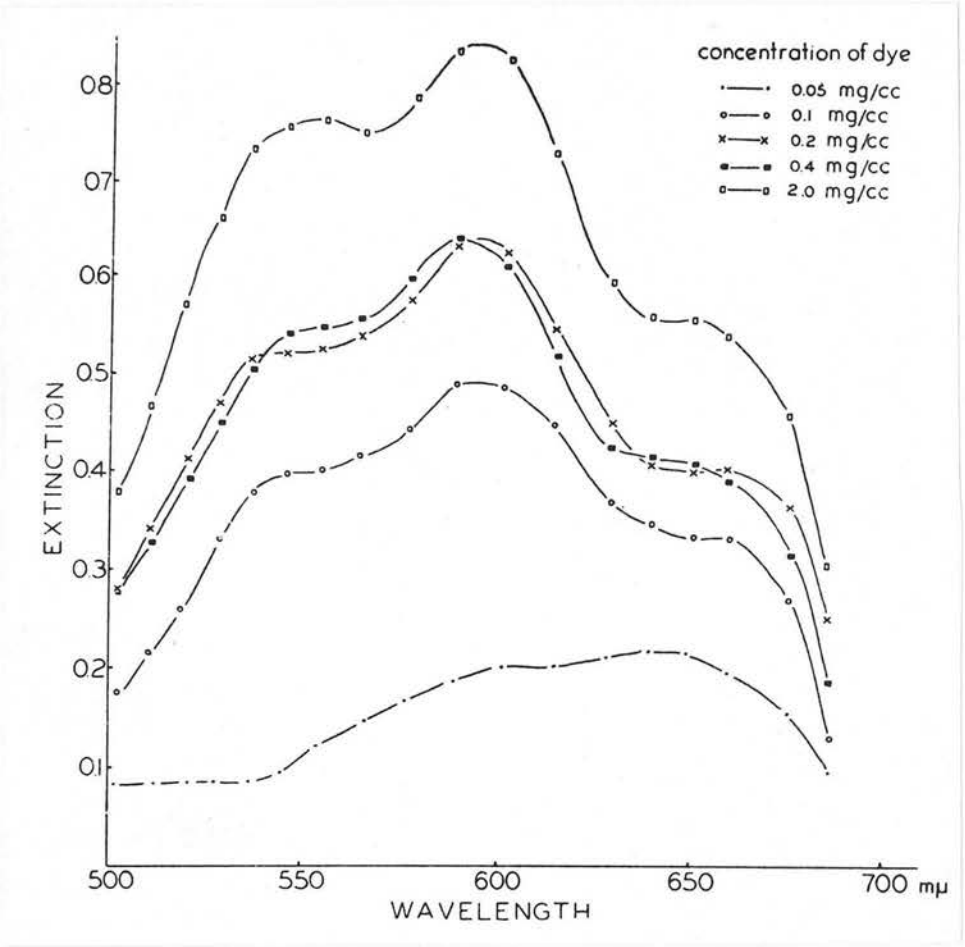


Fig.12. Effect of concentration of Azure B on RNA metachromasy. Corn-bud sections (8μ) stained with various concentrations of Azure B at pH 4; absorption measurements through microsporocyte cytoplasm. From Flax and Himes (1952).

perfect correspondence between the two sets of measurements. This type of comparison does much to increase one's confidence in the methods used.

When the conditions necessary for accurate microphotometric measurement of stained RNA are considered, it is not surprising that of all the basiphilic stains available very few can be used for its quantitative estimation. In fact, Azure B is the only one that has been used to any extent, and even with this stain caution is needed in interpreting the results.

Azure B is a metachromatic stain (Fig.11), its absorption characteristics depending on concentration of dye, nature of substrate (RNA,DNA, or mucupolysaccharides), pH and temperature. Under certain conditions, described by Flax and Himes (1952), it is considered specific for nucleic acids, and can be used to differentiate between DNA and RNA, the former staining blue-green and the latter purplish-blue. In tissues, as opposed to in vitro, it appears to stain RNA orthochromatically at least within the range of concentrations investigated. (Fig. 12). This is thought to be due to the absence of unbound dye, which in stained sections is washed away during differentiation. Swift (1955) reports that Flax found a good correlation between intensity of dye bound and the ultraviolet absorption of the cytoplasm of mouse oocytes, so it would seem that, at least under certain conditions and with certain materials, Azure B intensity may be regarded as bearing a stoichiometrical relation to RNA. Where DNA is likely to interfere, it can be removed enzymatically before staining. RNase-treated controls may be used to

check for unspecific staining, such as that caused by absorption to cell surfaces.

Swift and his school have used Azure B to investigate the relationships between nucleolar, chromosomal and cytoplasmic RNA in several systems, including developingoocytes and regenerating liver and during pollen formation (Swift et al., 1956; Woodard, 1958), and plant tumour growth (Rasch et al, 1959). The staining of chromosomal RNA was found to be enhanced after the removal of protein; this demonstrates the possibility that changes in dye binding do not necessarily reflect changes in the amount of RNA present, but may be due to an alteration in the RNA-protein relationship. Swift (1958) combined Azure B staining with autoradiography and calculated the relative specific activities of nuclear, nuleolar, and cytoplasmic RNA of Triturus liver and pancreas, on the assumption that microphotometry gave an accurate measure of the amount of RNA concerned. He found the highest specific activity in the extra-nucleolar RNA, the next highest in the nucleolus, and the lowest in the cytoplasm. This result is not in agreement with that of McMaster-Kaye and Taylor (1958), who carried out similar experiments on Drosophila salivary glands. They estimated the concentration of RNA in the chromatin, nucleolus and cytoplasm from the concentration of P^{32} attained after several hours of feeding on labelled food. They found that all three fractions reached the same level of maximum specific activity, although this was reached at different rates, the nucleolus reaching its maximum about three hours after feeding, while the other fractions were labelled more slowly. Difference in timing in the experiments of Swift and of McMaster-Kaye and Taylor may contribute to the discrepancy in the results.

In the study now to be described, the intensity of staining of S.pombe with Azure B has been measured with two purposes in mind. Firstly, it was hoped to evaluate the usefulness of Azure B staining as a quantitative method for RNA measurement in this material by comparing the results with those obtained by U.V. microspectrophotometry. At the same time, it was hoped that Azure B staining might throw some light on a problem suggested by Dr. J.M. Mitchison. As was mentioned above, the increase in length of S. pombe cells grown at 28°C (and also at 33°C) stops about three-quarters of the way through the life-cycle, and the volume of the cell remains constant between cell-plate formation and cleavage. The dry mass of the cell continues to increase during the constant volume stage, and as the laying down of the cell plate accounts for only 24% of this increase (Mitchison,1957), the concentration within the cell necessarily increases. If the cells are grown at 17°C, however, the mean generation time is increased to 10 hours, the cells become elongated (Fig. 13), and the dry mass curve is parallel to the volume curve, so that during the constant volume stage there is no increase in concentration; in fact, as the cell plate is formed during this time, the dry mass and concentration of the cell contents must decrease slightly. It was thought that if this difference in the growth pattern of cells cultured at 33°C and 17°C were due to an alteration in the pattern of RNA synthesis, the alteration might show up as a variation in the intensity of staining.

Material.

Cultures of S. pombe were grown at 33°C as described in the section on U.V. microspectrophotometry. Log phase cultures at 17°C were obtained by inoculating 10 ml. sterile wort broth with 0.2 ml.

of stock stationary culture and incubating in a water-bath at 17°C for 40 hours. The inoculations were timed so that the cultures grown at the two temperatures were in the log phase of growth at the same time.

Method.

The cells were concentrated by centrifugation at 1500g and smeared on glass No. 1 coverslips, Excess medium was allowed to drain to the edge of the coverslip and was blotted with Kleenex tissue. The smears were fixed by freeze-substitution so that the results would be comparable to those obtained by U.V. microspectrophotometry on similarly fixed cells. After hydration, half the smears were treated as controls, being incubated for 1 hour at 40°C in ammonium carbonate-ammonium acetate buffer, pH 7.6, containing 0.4mgm. RNase per ml. The remainder were incubated in buffer only. All were then washed with potassium acid phthalate buffer, pH 4.0, and stained as described by Flax and Himes (1952) in Gurr's Azure B (MacNeal) at a concentration of 0.2 mgm. per ml. of the same buffer for three hours at 40°C. The smears were then washed briefly in distilled water, differentiated overnight in tertiary butanol to remove adsorbed stain, (Michaelis, 1947), and mounted in Euparal. Throughout this procedure, smears from 17°C and 33°C cultures were treated back to back so that the conditions were as similar as possible and measurements of stain amounts on corresponding smears were comparable.

In preliminary experiments it was found that there was little to choose between Carnoy fixation (the method used by Flax and Himes) and freeze-substitution as far as shrinkage of the cells was

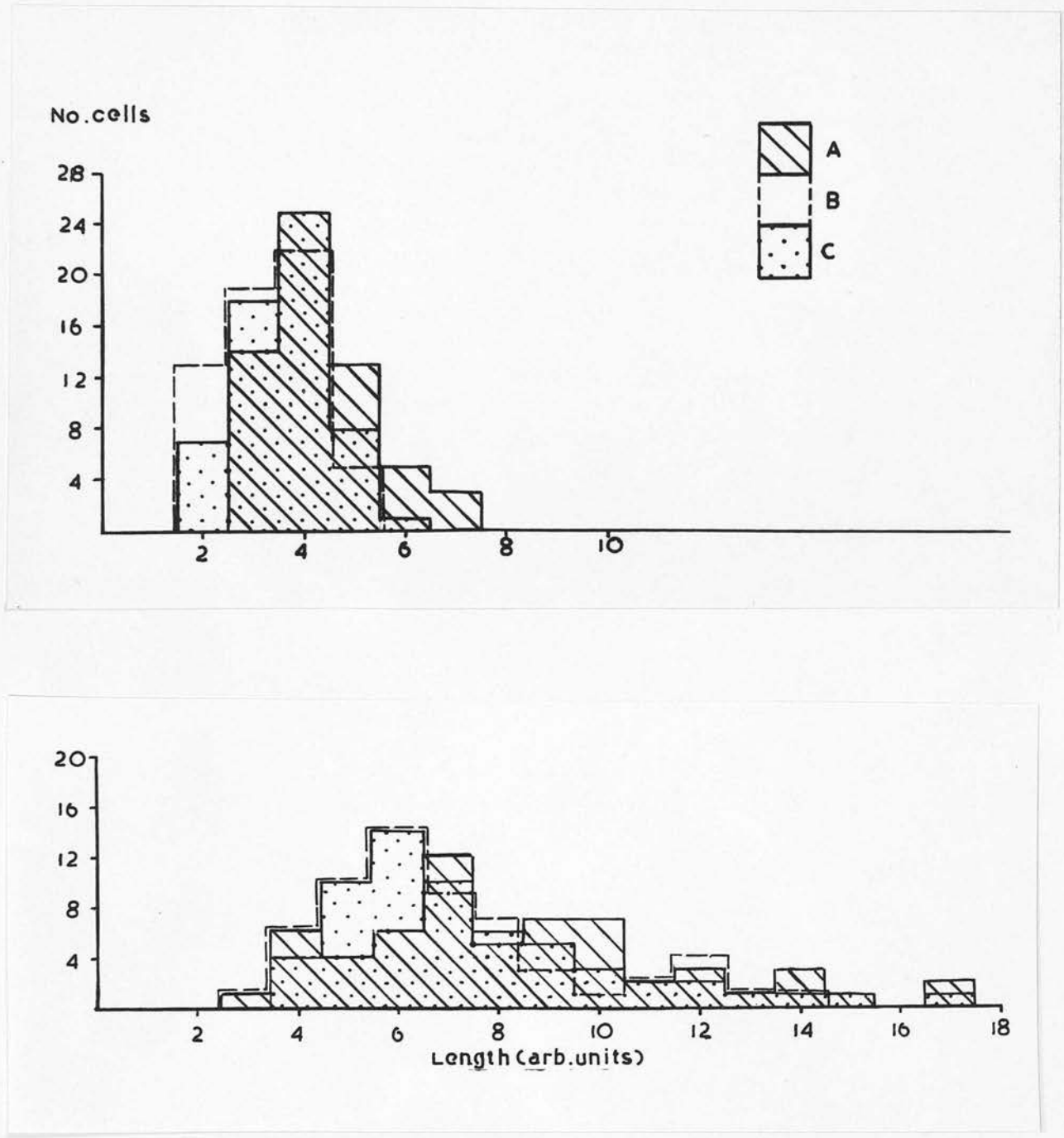


Fig.13. Length distribution in cells of S.pombe from log phase cultures grown at 33°C.(above) and 17°C.(below).

A unfixed

B Frozen-substituted, stained with Azure B and cleared with Euparal

C Carnoy fixed, stained with Azure B and cleared with Euparal.

concerned (Fig.13): both types of fixation resulting in about 30% shrinkage in width. Clearing with xylol appeared to shrink the cells still more so the use of a clearing agent was avoided by mounting in Euparal. Cells dehydrated with ethanol after staining were coloured faintly or not at all, confirming Swift's (1955) observation that Azure B is soluble in the lower alcohols: hence the use of tertiary butanol for differentiation. The specificity of Azure B for RNA under these conditions was shown by the total lack of staining of the RNase-treated controls smears. It was virtually impossible to see the cells except under phase-contrast.

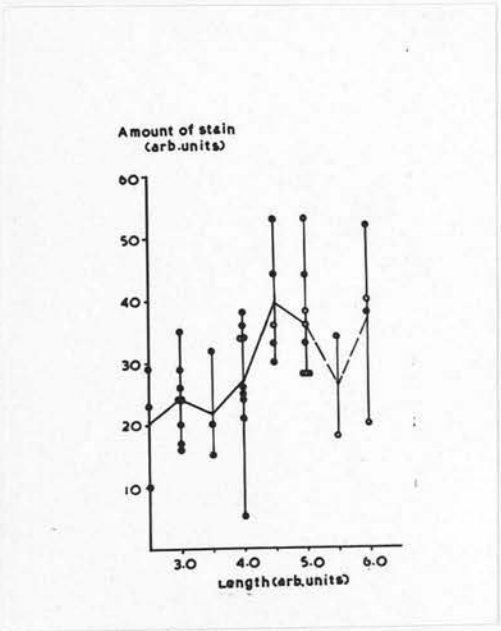
Amounts of stain in the individual cells were measured on the scanning and integrating microphotometer designed by Deeley (1955), using a mercury arc source with a Wratten 77 filter to isolate monochromatic light of wavelength 546 mμ. The cells were neither so densely stained nor so inhomogeneous that the use of the crushing condenser was necessary. The amount of stain in about 40 cells on each slide was measured, the average of three readings being taken for each cell: the length of each cell in arbitrary units was measured with a micrometer eye-piece.

Results and Discussion.

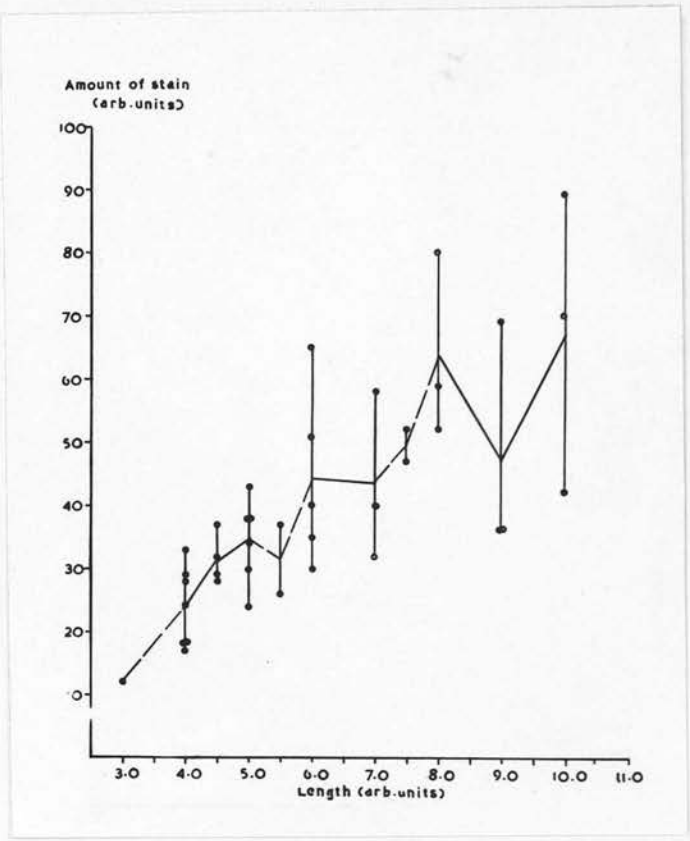
The results are presented graphically in Figs.14 & 15.

1. Comparison of results obtained by Azure B staining and U.V. microspectrophotometry of cells grown at 33°C.

Both sets of results show a large scatter about the mean at any particular stage in the cell cycle. Some of this scatter is undoubtedly due to biological variation: that this is present is obvious from the differences in length of individual cells with cell



(a)



(b)

Fig.15. As 14.

plates, i.e., cells at the constant volume stage. Apart from this biological variation, scatter about the mean will depend on the accuracy of the method used. Comparing Figs.14a and 15a with Fig. 10. it can be seen that the scatter is larger in the Azure B measurements than in the ones obtained by U.V. microspectrophotometry. This means either that the two methods are measuring different RNA fractions i.e. that part of the RNA has been removed during the staining procedure, or, assuming that the two methods are measuring the same material, that the Azure B method is less accurate than the U.V. microphotometric one, either due to lack of stoichiometry in the dye-substrate relationship, or to errors inherent in the method of measurement. In any case, it would seem that an ultra-violet microphotometric method is more satisfactory than Azure B staining for RNA measurement in this material under these conditions. U.V.methods also have the advantage of the possibility of measuring the absolute amount of RNA, but on the other hand, in choosing a method to investigate a particular problem, the availability and cost of the apparatus necessarily plays a part, and instrumentation for microphotometry in visible light is simpler and cheaper. Determination of stain amounts is also more rapid than the measurement of total absorbance of U.V. radiation except with very complex and costly apparatus.

If one considers the average curves obtained by both methods, an approximate doubling in amounts of U.V. absorbed and stain measured is apparent. Owing to the scatter about the means mentioned above, it is difficult to draw definite conclusions about the shape of the

curve, though perhaps there is a slight suggestion of a positive curve with a falling off in the rate of RNA synthesis towards the end. This is more apparent in the U.V. measurements than in the Azure B ones.

In both sets of measurements, if a large part of the increase in dry mass during the constant volume stage were to be accounted for by a continued increase in the amount of RNA, one would expect a greater scatter about the mean of the values for cells with cell plates of any particular length (and therefore volume) than for cells without cell-plates. This does notappear to be so; this fact together with the slight drop at the end of the curve, suggests that the increase in dry mass during the constant volume stage is due to other factors.

2.Comparison of Azure B measurements on cells grown at 17°C and 33°C

Assuming that the amounts of Azure B are stoichiometric with the amounts of RNA in the cells, from the curves in Figs. 14 and 15, it is apparent that the increase of RNA bears the same relationship to the increase in length in cells grown at 17°C and at 33°C, although increase in both length and RNA amounts proceeds at a slower rate at the lower temperature. In the cells grown at 17°C, the RNA continues to increase throughout the life cycle although the cells may attain twice the length of those grown at 33°C. It therefore appears that the division mechanism is slowed down more than the mechanism. associated with protein and RNA synthesis. The variation between individual cells is too great to justify speculation on the pattern of RNA synthesis after cell plate formation: it may be that the method is less accurate for measuring amounts of stain in these

elongated cells owing to the very large area of background which must be included with the specimen in the scanned area. Ris and Mirsky (1950) found that in measuring the amounts of Feulgen stain in small dense nuclei, the increase in non-absorbing area obtained by different settings of the image plane diaphragm caused an increase in the calculated extinction. On the other hand, the increased variation in these very elongated cells may be a biological one, as slight differences in RNA synthesis rates between individual cells with different growth rates would result in a larger variation between the amounts of RNA in the cells concerned, the longer the duration of interphase.

2. OTHER CYTOCHEMICAL METHODS.

Two other methods for the quantitative estimation of RNA in individual cells remain to be mentioned. The first of these is X-ray historadiography, which, like the techniques described above, depends on the absorption of radiation by cellular material, and which was developed by Engstrom and reviewed by him. (Engstrom, 1956, 1959; Engstrom and Lindstrom, 1958). The second method is interference microscopy, which differs from the other methods in that it does not measure the absorption of radiation, but its optical retardation. As this is dependent on the amount of material present, the optical retardation can give a measure of dry weight, as does X-ray historadiography, Interferometry has been reviewed by Barer (1956, 1959), Davies (1958), and Davies et al. (1954).

Both methods were developed for the measurement of the dry weight of cells, or parts of cells, and since by far the greater part of the dry mass of most living material consists of protein, dry weight is usually equated with protein. When combined with specific extraction techniques, they can also be used for the estimation of nucleic acids, at the same time permitting a determination of the concentration of the nucleic acids on a dry weight basis. Interference microscopy has the great advantage that living material can be used (although fixation is usually necessary before extraction techniques can be applied) and in this is to be preferred to historadiography, as here the specimen must be frozen-dried and the measurements carried out in vacuo.

Results obtained by the two techniques have been compared by Davies, Engstrom and Lindstrom (1953) and Davies and Engstrom (1954), working on bone and other mammalian tissues, and by Kimball et al.

(1959), in a study of the growth of Paramecium between divisions, with good agreement. The measure of agreement found by Nurnberger, Engstrom and Lindstrom (1952) in the measurement of protein amounts in nerve cells by historadiography and U.V. microspectrophotometry appeared to depend on the method of fixation for the latter method, Carnoy fixation giving the best correspondence between the two sets of results. Davies et al. (1954) measured the dry weight of individual ram sperm by interferometry: this value, combined with that for the amount of DNA found by Walker and Yates (1952) using a U.V. absorption technique, showed that 40% of the dry weight of the sperm consisted of DNA. This agrees well with the value of 45% obtained by biochemical technique. As mentioned in the introduction, King's (1959) study of the cytoplasm of mouse ascites tumours cells is another example of the comparison of results reached by two different methods. In this case, the value for total dry mass obtained by interference microscopy corresponded well with the sum of the values for protein and nucleic acids determined from U.V. absorption measurements.

Interferometry combined with extraction techniques was used by Davies, Deeley and Denby (1957) in an investigation of the composition of sperm. Lipid and nucleic acid were successively extracted, the dry mass of the sperm being determined before and after each treatment, values for the amounts of lipid, nucleic acid and protein thus being obtained. Small molecular weight substances were ignored, but the error thus introduced is negligible. Stenram (1958 a and b) measured the concentration of rat liver nucleoli before and after RNase extraction. The concentration was the same in both

protein-fed and starved rats, but as the nucleoli were larger in the former, the amount of RNA was greater. Changes in nuclear protein and RNA content of Cyclops eggs during mitosis were investigated by Stich and McIntyre (1958). After removal of lipid most of the dry weight ofthe nucleus as determined by historadiography was due to protein, and RNase digestion gave a reduction of 4-6% of organic matter. They also measured the RNA semi-quantitatively by cytophotometric absorption after gallo-cyanin chromalum staining, and, taking these results together with those obtained by X-ray absorption, concluded that RNA accumulated in the nuclear sap at the end of interphase. A similar semi-quantitative study on the isolated mitotic apparatus of the sea-urchin Strongylocentrotus purpuratus was carried out by Rustad (1959), but in this case interferometry was used to determine changes in dry weight. Staining reactions showed that RNA was lost from the aster region during mitosis.

CONCLUSION TO PART I.

The use of different techniques to investigate the pattern of RNA synthesis in the fission yeast S. pombe has emphasised the importance of the point made in the introduction: namely, that whenever possible, several cytochemical techniques should be employed to investigate the same problem, and that the results obtained in this way should be compared with those obtained biochemically. The importance of this is illustrated bythe U.V. work on unfixed and frozen-substituted cells which also makes clear the need for quantitative investigation on the effects of each experimental procedure on the cells, and for the use of unfixed cells as a reference wherever possible.

For the solution of any particular cytochemical problem, both the techniques and the material available should be taken into account. For instance, S. pombe is suitable material for the cytochemical investigation of the synthesis of RNA, but the cell is not a suitable shape for accurate investigation with microphotometers of the usual design i.e. with a circular diaphragm limiting the scanned area. Neither is it suited to an extraction method, owing to its small size and small RNA content, and possibly owing to its possession of a thick cell wall. For this material, which contains a high concentration of RNA and a high RNA; DNA ratio, 'in situ' U.V. microspectrophotometry is the most suitable technique. On the other hand, for the investigation of the same problem in large inhomogeneous cells with a high RNA content, large nuclei and without thick cell walls, Edstrom's extraction technique is particularly suitable.

If a reliable stoichiometric stain for RNA could be found, microphotometry in visible light could become as useful a method for RNA measurement as it is at present for the measurement of DNA. Azure B may satisfy this requirement for some materials : the ideal way to test any particular case would be to measure the absorption of stained material (after the removal of other U.V. absorbing substances) at 265 mμ and at the appropriate wavelength in visible light, as was done by Walker and Richards (1957) in an investigation of the stoichiometry of Feulgen staining for DNA in sperm heads.

The U.V. microspectrophotometric work on S. pombe has also demonstrated the possible inhomogeneity of RNA within the cell, and the need for techniques for the investigation of the fractions. This

is the aspect of RNA estimation which is dealt with in Part II.

PART II. THE HETEROGENEITY OF RNA.

Part I is concerned with the RNA of the cell as a whole, but the RNA within the cell is not homogeneous in its distribution, its composition or its biological activity. The applicability of quantitative cytochemical methods to these three aspects of the RNA of individual cells will now be examined.

i) Distribution.

Any of the methods described in Part I may be used to study the distribution of RNA in the cell provided allowances are made, if necessary, for the contribution made by the RNA of parts of the cell other than that being measured. For example, measurement of the absorption of U.V. radiation by the nucleus may involve a correction for absorption by the cytoplasm above and below it, the size of the correction depending on the shape of the cell. For this reason, it may be preferable to work with isolated cell organelles (e.g. Leuctenberger et al., 1952) but here there is a risk of loss or alteration of RNA during isolation. The use of Edstrom's extraction method on parts of cells necessarily involves the isolation of the cell organelle concerned, either by micro-dissection or by the usual biochemical method of homogenization and differential centrifugation. In a few exceptional cases, it is possible to centrifuge single cells and to stratify the components, which can then be isolated by cutting the cells at appropriate points. This has been done in cytochemical investigations by Harvey (1940) and Immers (1960) on sea-urchin eggs, by Holter (1954) on the amoeba Chaos chaos, and by Zalokar (1960), working with Neurospora.

ii) Composition.

It might be expected that the RNA's of different parts of the cell would have different chemical compositions associated, for example, with the synthesis of particular proteins. It is also possible that the various fractions may change in composition during the life-cycle of the cell, or during pathological processes. Intercellular differences might be associated with morphological and/or functional differentiation. These differences, if sufficiently marked, would be shown up as variations in the proportions of the four bases (adenine, guanine, cytosine and uracil) and their corresponding nucleotides, i.e. as a change in the base ratio. The evidence obtained by biochemical techniques for such changes is conflicting. Elson and Chargaff (1955) could detect no difference between the nucleotide composition of RNA prepared from different organs of the same species, or even between species, when the total RNA was investigated, but when subcellular fractions were examined, the nuclear and cytoplasmic RNA's of the rat were found to have different compositions. Kleinschmidt (1959) could not detect any significant difference in the base ratios of nucleic acids from normal and neoplastic mouse and chicken tissues, but did not investigate the cellular fractions. A similar investigation on normal and tumour tissue was carried out by Kit (1960), who also examined the base ratio of RNA from the microsomal and two nuclear fractions. For any given tissue, the base ratios of the fractions resembled each other and that of the total RNA of the tissue, and Kit found that the base composition of RNA from, for example adult, newborn and neoplastic mouse tissues was similar, and so was that from at least

two fractions of spleen and the lymphomas examined. Low (1958), working on several species of alga, and Bather (1958), investigating Rous sarcoma viruses, found no significant variations in the composition of the RNA's of the different organisms they examined. In contrast to this is the work of Gierer and Mundry (1958), who found they could produce mutants of tobacco mosaic virus by chemical alteration of its RNA *in vitro*, and that of Minigawa (1957), who induced a quantitative change in the RNA of the yeast *Saccharomyces ellipsoideus* by growing it in a copper-containing medium. The change made the yeast less susceptible than the parent strain, to the copper poisoning. Levenbook et al. (1958) and Travaglini et al. (1958) found that the composition of the N.A's of unfertilised eggs of *Drosophila melanogaster* was affected by their genetic constitution: XXY type RNA contained a higher proportion of adenine than XX RNA. The composition of acid-soluble compounds was also affected.

Other authors have investigated the base composition of the RNA of micro-organisms growing in synchronous culture, to see if changes in the base ratio could be detected during the life cycle of the cells. Scherbaum (1957b), working on *Tetrahymena pyriformis* synchronised by heat shocks, could detect no such differences, although the bases did show different rates of incorporation of ^{32}P at different stages in the life cycle. *Chlorella ellipsoides*, synchronised by variations in light intensity, was used for a similar investigation by Iwamura and Myers (1959). In this case the guanylic acid content of the RNA was found to be greater during

active increase in cell mass in the light than during dark incubation, when no growth occurs but DNA synthesis and cell division are able to take place. It is interesting to compare these results with those of de Lamirande et al. (1955, 1958), who found that the composition of RNA from subcellular fractions of rat liver was more homogeneous in tumorous than in normal liver, the chief difference being the high proportion of guanylic acid in the former, especially in the supernatent fraction. The composition of regenerating liver RNA was intermediate between that of normal and tunorous liver.
De Lamirande et al. suggest that the high guanylic acid content may be associated with a high rate of mitosis, but as in this case a high rate of cell division also implies a rapid increase in cell mass, this result could also mean that RNA with a high guanylic acid content is associated with active growth : this interpretation would bring it into line with the result of Iwamura and Myers. One wonders if there is any connection between these results and the fact that guanylic triphosphate is an essential constituent of cell-free systems which can incorporate amino-acids into protein.

This type of problem, concerned with chemical changes in RNA during the growth and division of the cell, is one in which a cytochemical approach would have an advantage over a biochemical one, but it is also one which is difficult to attack with cytochemical techniques. The only method at present available for the investigation of RNA composition on a cellular scale is the microphoresis method of Edstrom (1956) referred to earlier. This method is applicable to amounts of RNA in the range of 100-1000µµg.,

which is above the normal mammalian cell content (except for nerve cells, which do not grow or divide in the adult mammal). It could probably be used with success on some Protozoa, although even then, if the RNA composition of organelles such as the nucleus or nucleolus were to be investigated, it might be necessary to pool the appropriate organelles from several cells at the same stage.

iii) Biological activity.

The basis of the investigation of the biological activity of the RNA of cellular fractions is in principle the same; namely, the incorporation of RNA precursors labelled with radioactive isotopes. In experiments on a biochemical scale, the isotope may be incorporated in vivo and the relative specific activities in the fractions determined after tissue homogenisation and isolation of the fractions by differential centrifugation, or the fractions may be isolated first, and then incubated with the isotope in vitro. The latter approach lends itself to the criticism that the experimental conditions may cause the course of incorporation to be abnormal, and with both there is the danger always present in such experiments; that of the possible redistribution of RNA during experimental procedures. It is always to the good, therefore, if results from such experiments can be supported by evidence obtained by cytochemical means, in this case, by autoradiography. For example, nuclear RNA has been shown by several means to consist of at least two fractions with different metabolic activities. Allfrey and Mirsky (1957) studied the incorporation of labelled RNA precursors into the RNA of nuclei isolated from calf thymus and found that the nuclear RNA was

metabolically far more active, and showed greater susceptibility to inhibitors of RNA synthesis, than did the other fraction. Both fractions had similar base ratios. Osawa et al. (1958) obtained similar results, using the in vivo incorporation method with subsequent isolation of fractions.

A cytochemical investigation of RNA in Drosophila salivary glands was carried out by McMaster-Kaye and Taylor (1958), using autoradiography for the localization of the labelled precursors. They also found two metabolically distinct nuclear fractions localized in the nucleolus and chromosomes respectively, the nucleolar RNA reaching maximum activity far more quickly than that of the chromosomes, whose activity was similar to that of the cytoplasm. There is recent evidence, summarised by Sirlin (1960), that the nucleolar RNA itself exists as at least two fractions differing in metabolic activity.

In the preceding pages, no mention has been made of the heterogeneity of cytoplasmic RNA itself, because as yet there are no methods available for the investigation of the cytoplasmic fractions on a cellular scale. The experimental work to be described later is concerned with an attempt to develop a technique which could be adapted for use on individual cells for the separation of the two main fractions : since this must necessarily be based on the physical and chemical nature of the cytoplasmic RNA, it is relevant to consider first what is known about its organization.

CYTOPLASMIC RNA

i) Particulate RNA

Contemporaneously with the work of Caspersson and Brachet on the RNA of individual cells in the late 1930's, Claude found that it was possible to obtain by the homogenisation and fractionation of many types of tissue, a fraction containing up to 60%* of the total RNA in the form of particles 500-2000 A in diameter. These particles consisted of ribonucleoprotein together with a high concentration of phospholipid, and Claude (1943) called them microsomes. Later, Palade and Siekevitz (1956) separated the phospholipid from most of the RNP with sodium deoxycholate and found that the RNP was in the form of particles 150-200 A in diameter, and that they contained 80-90% of the RNA present in the original microsomes. At the same time, electron microscope studies of cellular ultrastructure, many of which were carried out by Palade and his co-workers, demonstrated the presence of particles of this size mostly attached to membraneous structures forming a network throughout most of the cytoplasm, termed the endoplasmic reticulum. They concluded that microsomes consisted of fragments of this reticulum with its attached particles and that treatment with deoxycholate dissolved the former, owing to its lipoprotein nature, freeing the RNP particles. Roberts (1958) has suggested that RNP particles with a sedimentation constant of 20-100S, corresponding to the particles of size 100-200 A in diameter demonstrated by the electron microscopists, should be termed 'ribosomes' to avoid confusion with the microsomes themselves. This term will be adopted here.

The extent and nature of the endoplasmic reticulum depends on the

---

* In bacteria, the percentage of cytoplasmic RNA recovered in this fraction may be as much as 90% (Loftfield, 1957).

type of cell. In cultured fibroblasts, an extensive reticulum was found, parts of which had ribosomes attached and parts of which were smooth. Exocrine pancreatic cells have a very extensive reticulum, most of which is covered with ribosomes, and the cells also contain a large number of free particles. Rapidly growing cells such as erythroblasts and undifferentiated epithelial cells have a poorly developed reticulum and a large number of free ribosomes, while the situation is reversed in non-dividing cells such as leucocytes and seminal epithelium (Palade, 1958). Cells of two types of liver tumour showed very poor development of the endoplasmic reticulum compared with normal cells, but contained large numbers of free ribosomes (Howatson and Ham, 1955). In micro-organisms there is little or no reticulum, most of the numerous ribosomes occurring free in the cytoplasm. (Chapman and Hillier, 1953; Chapman, 1959; Agar and Douglas, 1957; Hashimoto et al., 1958).

It was found that the types of cell which contain a large number of particles were also those which Caspersson and Brachet had shown to contain large amounts of RNA which they claimed was associated with protein synthesis, and the suggestion that the ribosomes are the cytological basis of protein synthesis has been amply supported by biochemical evidence, which has shown that they are the site of the incorporation of amino-acids into protein.

Siekevitz (1959) has suggested that the function of the endoplasmic reticulum is in assisting in the removal of newly synthesised protein from secretary cells. Indeed, in the exocrine cells of guinea pig pancreas (though not in that of the rat), the

secreted enzymes appear to condense into intracisternal granules which are carried to the Golgi region where they form zymogen granules. This theory would account for the lack of an extensive reticulum in micro-organisms, where the proteins produced are all used within the individual cell.

A recent paper by Hanzon et al. (1959) has cast doubt on the existence of ribosomes in the untreated cell. This doubt is based on the failure of the authors to demonstrate the presence of the particles by electron microscopy after fixation by freeze-drying instead of by conventional osmium fixation. Particles were demonstrated in the appropriate cell fraction isolated by differential centrifug ation and then frozen-dried, and Hanzon et al. suggest that the RNP is not organized as 150 A particles in the living cell, but rapidly condenses to this form when the internal milieu of the cell is changed by such procedures as chemical fixation and homogenisation. Nevertheless, the universal occurrence and functional significance of such particles has been so amply demonstrated that, even if they are strictly speaking artifacts, their study has been well justified.

Physico-chemical studies have been carried out on ribosomes isolated from many different types of cell. Their properties depend on the physiological state of the organism and on the experimental procedures used, but in much of the experimental material examined there appears to be a basic component consisting of spherical particles 100-200 A in diameter, of molecular weight 4×10^6, with a sedimentation constant of 70-80S and containing approximately equal amounts of RNA and protein. Such particles have been isolated from

Escherichia coli (Bolton et al., 1958; Elson, 1959; Hall and Slayter, 1959; Roberts et al., 1959; Tissières et al.,1959; Wagman and Trawick, 1958)*, from Saccharomyces cerevisiae (Ashikawa, 1958 Chao, 1957;) Chao and Schachman, 1956; Wolfe, 1956), from Azotobacter vinelandii (Gilchrist and Bock, 1958), from Neurospora (Chao, 1957), from pea seedlings (T'so, 1958), from rabbit reticulocytes (Dintzis et al., 1958), from calf liver (Hall and Doty, 1959), and from several other types of mammalian tissue (Petermann et al., 1958a). In most cases, the picture is complicated by the presence of smaller particles. The relative amounts of the different components in E.coli have been shown to vary with the composition of the growth medium (Bowen, Dagley and Skyes, 1959; Dagley and Sykes, 1958), and Wade and Morgan (1957) have shown that certain components are present in greater amounts in dividing than in stationary cells. A similar result was obtained by Ashikawa (1958) with S.cerevisiae, particles with a lower sedimentation constant appearing during the lag phase, but being absent from stationary and starving cultures. On the other hand, Britten et al., (1960) found no great difference in the sedimentation pattern of ribosomes from different stages of the cell division cycle in synchronous cultures of Alcalogenis faecalis. Petermann and her co-workers (Petermann and Hamilton, 1952; Petermann and Hamilton, 1958b; Petermann et al.,1958), using rat liver, obtained a sedimentation pattern which varied with the physiological state of the tissue. They found a larger proportion of the smaller components present in

---

* Other authors find the 'basic component' of E.coli to sediment at 40S (Bowen,1959; Bowen,Dagley & Sykes,1959; Dagley & Sykes,1958. Also see Loftfield,1957). This may be due to differences in the medium.

regenerating liver than in normal liver, especially during the period of most rapid cell division.

In vitro, the size of the particles can be altered by changes in the ionic environment (alteration in the intracellular environment may be responsible for the variations mentioned above). The concentration of magnesium is especially important for the maintenance of the integrity of the 80S particles; if this is below a certain value reversible dissociation into smaller particles occurs (Bolton et al., 1958, Chao and Schachman, 1956; Gilchrist and Bock, 1958; Hall and Slater, 1959; Huxley and Zubay, 1960; Petermann et al.,1958; Tissières and Watson, 1958; Tissières, et al., 1959; Wolfe, 1956). Tissières et al., (1959) and Huxley and Zubay (1960) found that raising the concentration of magnesium from that in which the 70S particles were stable caused aggregation of the 70S particles to form dimers with a sedimentation constant of 100S. In some cases, it has been shown that the smaller particles have the same composition as the 'parent' particle as regards base ratio (Spahr and Tissières, 1959), and protein: RNA ratio (Elson and Tal., 1959; Roberts et al., 1958; T'so, 1958; Petermann and Hamilton, 1958b).

From physico-chemical studies on calf liver ribosomes and on RNA isolated from them, Hall and Doty (1959) have concluded that in both the RNA consists of polynucleotide chains with helical regions, and that a large percentage of the nucleotides are linked by hydrogen bonds. This percentage is 40% in the free RNA and 30% in the RNP and Hall and Doty suggest that in the ribosome the number of sites represented by the 10% difference may be available for participation

in the assembly of protein molecules in some way, perhaps for bonding with soluble RNA.

As well as RNA in the ribosomes, the presence of RNA in other cytoplasmic particulates, the mitochondria, has been claimed (Birbeck and Reid, 1956; Simpson et al., 1957). Other authors (e.g. Novikoff, 1956) have been unable to detect any RNA in the mitochondria and suggest that reports that it is present are due to contamination of the mitochondrial fraction with microsomes. Even if present, it is unlikely to comprise a large proportion of the cytoplasmic RNA.

ii) Soluble RNA.

After the sedimentation at 105,000g of the ribosomes from cell homogenates, there remains a certain amount of cytoplasmic RNA in the supernatent. This was called 'soluble' RNA by Brachet and Jeener (1944). Since the recent elucidation of its function, it has been termed 'transfer' RNA (Hoagland, 1959), but since I am more concerned here with distribution than with function, the older term will be retained.

The proportion of soluble to ribosomal RNA varies greatly among different preparations, and although some of these variations are likely to represent real biological differences, no doubt others are caused by the methods of tissue homogenisation and separation of the fractions. Anderson (1956) found that 10% of the RNA of rat liver was in the non-sedimentable fraction and suggests that the high values

obtained by some other workers were due to the washing of loosely adsorbed RNA from RNP particles. Quattrone (1959) suggests that the values of 19-33% obtained for SRNA from the same material by various workers may be accounted for by enzymatic degradation of the ribosomal RNA during fractionation. A comparison of four different combinations of homogeniser and extraction medium for the separation of subcellular fractions of calf thymus was carried out by Hess and Lagg (1958). The amount of SRNA varied from 76-94% of the total RNA. They relate this high value to the poorly developed endoplasmic reticulum of the thymocyte, but attribute the variation to the method of fractionation. Maruyama and Lark (1959) found 50-60% of the total RNA of Alcalogenis faecalis to be non-sedimentable and suggest that this value may be high owing to the low concentration of magnesium in the medium. Zalokar (1960), working on Neurospora hyphae, found appreciable amounts of RNA in the supernatent fraction of homogenised hyphae, but if living hyphae were centrifuged whole, he could not detect RNA in the supernatent layer by cytochemical staining technique. If this result were to be confirmed by U.V. methods, it would present strong evidence for the view that SRNA is not morphologically distinct from the particulate RNA in the living cell but is easily detached from it by homogenisation and fractionation techniques.

Other variations have been accounted for by different physiological and pathological states of the cells in the tissues concerned. For example, Lowe and Lehninger (1955) found that in the liver of cortisone-treated rats almost all the RNA was in the supernatent

fraction. They suggest that cortisone may effect the degree of polymerization of the RNA. Tumour cells and other actively growing and dividing cells contain a higher proportion of non-sedimentable RNA than corresponding 'resting' cells (de Lamirande, 1955); Brachet, 1957). In exponential cultures of E.coli, Tissières et al. (1959) found that 10-20% of the total RNA was in the non-sedimentable fraction and this has been confirmed by the work of Lacks and Gros (1959).

Estimations of the molecular weight of SRNA prepared from various sources vary from 10,000-40,000 (Dunn, 1959; Otaka and Osawa, 1960; Tissieres, 1959; Weiss, 1958; Zamecnik et al.,1958). Tissieres (1959) concludes from a study of some of the physical and chemical properties of SRNA from E. coli that it is not bound to protein in cell-free extracts, but is present as free RNA. The situation appears to be more complex in mammalian tissues : Shigeura and Charg aff (1958) found that the SRNA of rat liver cytoplasm was capable of sub-fractionation. Only one fraction, which comprised 10-20% of the total SRNA, was not combined with protein, but this fraction was shown to have a higher specific activity (turn-over of ^{32}P) than the other three fractions combined, which consisted of relatively inert RNP.

The degree of hyperchromicity of SRNA after hydrolysis seems similar to that of particulate RNA (Doty et al., 1959; Tissières, 1959). It is thought (Tissières, 1959) that, as with ribosomal RNA, the nucleotides are bound together with hydrogen bonds.

Very small amounts of purine and pyrimidine bases other than

adenine, guanine, cytosine and uracil have been found in ribosomal RNA (Littlefield and Dunn, 1958a and b), but in SRNA these tend to occur in larger proportions (Otaka and Osawa, 1960; Dunn, 1959), although they still account for only a small percentage of the total amount of bases. Apart from these 'odd' bases, there is some evidence that the base ratio of SRNA differs from that of RNA in other cell fractions (Reid and Steven, 1958; de Lamirande, 1958; Tissières, 1959).

Because of the tendency for SRNA to form a larger proportion of the total RNA in cell populations which are dividing rapidly than in resting populations, some authors (e.g. Brachet, 1957) have suggested that SRNA may be concerned with mitotic activity. However, recent work has shown that the non-sedimentable RNA is concerned in the early stages of protein synthesis.

Incorporation studies by many workers using cell fractions isolated from various sources have shown that the first step in the incorporation of an amino-acid into protein is its 'activation' by a so-called activating enzyme to form an amino-acyl adenylate. The amino-acid is then transferred to the SRNA by a second enzyme; there is evidence for a specific SRNA for each amino-acid (Berg and Ofengand, 1957; Schweet et al., 1958; Lipmann et al., 1959). The enzymes, together with a third active at a later stage, and most of the SRNA, are precipitable at pH 5: the precipitate from the 105,000g supernatent is therefore known as the 'pH 5 fraction'. The soluble, or 'transfer' RNA carries the attached amino-acid to the ribosome where it may be deposited at an appropriate site, perhaps on an RNA

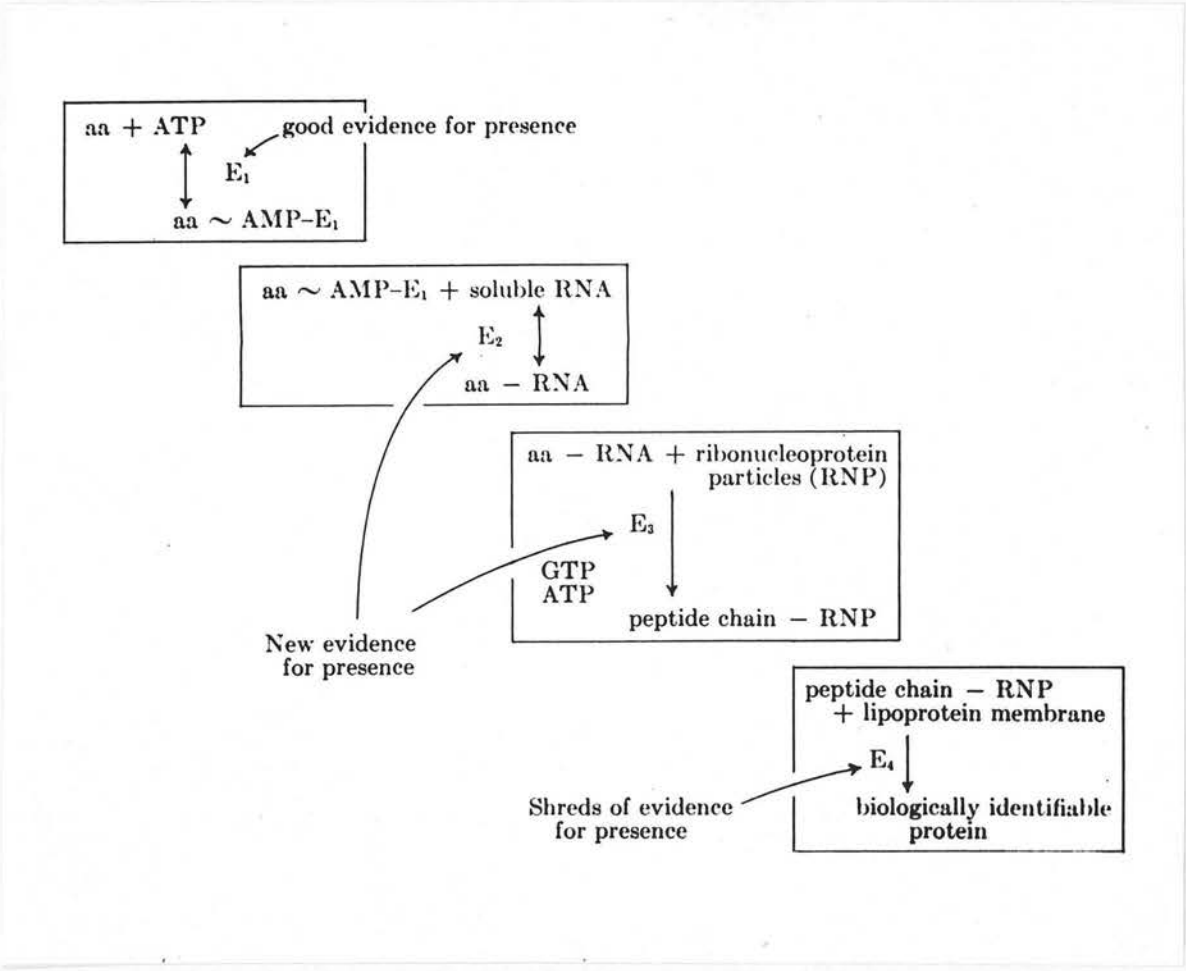


Fig.16. Diagrammatic scheme of the steps involved in protein biosynthesis. From Zamecnik et al. (1958).

template. Some or all of these reactions have been shown to occur in various in vitro systems provided with appropriate energy sources by Berg and Ofengand (1958), Hoagland and Zamecnik (1957), Hoagland et al., (1957), Lipmann et al., (1959), Mager and Lipmann (1958), Ogata and Nohara (1957), Schweet et al., (1958), Webster (1957), and Wiess (1958) and have been summarized by Zamecnik et al. (1958), from whom Fig. 16 is taken.

THE ORGANIZATION OF CYTOPLASMIC RNA IN SCHIZOSACCHAROMYCES POMBE

Materials.

As has already been mentioned, S. pombe is particularly convenient material for the quantitative investigation of RNA, owing to its relatively high RNA concentration and very low DNA:RNA ratio.

For the biochemical work now to be described, cultures containing 3-6 x 10^6 cells, i.e. cultures in the second half of their log phase, were used, unless it is stated otherwise. These were obtained by inoculating 750 mls. sterile 2% w/v Oxoid wort broth in one litre Erlenmeyer flasks with 3 mls. stock stationary culture and incubating for 16 hrs. at 33°C. The cells were spun down at 800g and washed twice with buffer containing 3.65 x 10^{-3}M. K_2HPO_4, 2.5 x 10^{-3}M. KH_2PO_4 and 1 x 10^{-3}M. $MgSO_4$. 7 H_2O. Cells prepared in this way were used for all the experiments in Part II, and, except where otherwise stated, the cells and cell fractions were always suspended in buffer of this composition, which will be referred to as 'ribosome' buffer, since it is similar in composition to that found by Chao and Schachman (1956) to maintain the integrity of the ribosomes of Saccharomyses cerevisiae. Cell fractionation experiments were carried out in collaboration with Dr. P.M.B. Walker, and the experimental procedure was based on that of Chao and Schachman.

Method.

A very thick suspension of washed cells was transferred to a cold (-14°C) Hughes bacterial press (Hughes, 1951), where they were broken by compression. An approximate estimate of the percentage of cells broken was obtained by counting broken and unbroken cells in a suitably diluted sample on a haemocytometer under phase contrast, the

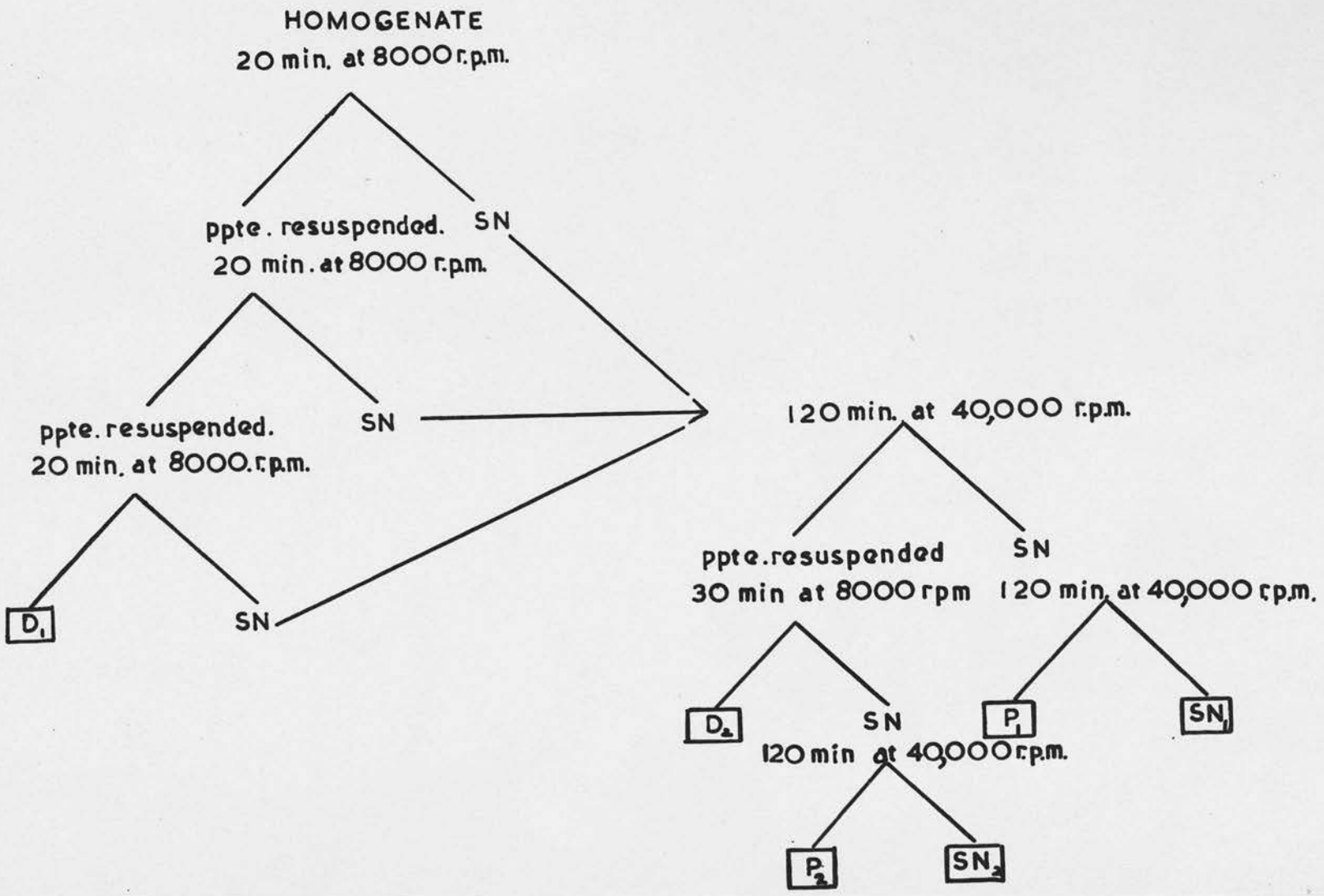


Fig.17. Procedure followed for the separation of cell fractions of S. pombe by differential centrifugation.
D_1 and D_2 were combined to form the DEBRIS fraction.

P_1 and P_2 were combined to form the PARTICLE fraction.

SN_1 and SN_2 were combined to form the SUPERNATENT fraction.

broken cells appearing dark. In counting broken cells, fragments larger than one half the size of a cell about half way through its life cycle were counted as a whole cell and smaller fragments were ignored. Unfortunately, with the method of disintegration used here, the number of cells broken never exceeded 50%. After a sample of the homogenate had been removed for RNA estimation, the remainder was fractionated by ultracentrifugation. At all stages of the fractionation, the experimental material was kept between 0° and 4°C to minimise spontaneous breakdown of RNA. Fractionation was carried out in a Spinco Model L preparative centrifuge, using a No. 40 rotor. (See Fig. 17).

The RNA and acid-soluble nucleotides in three aliquots of the homogenate and of each cell fraction (debris, particles and supernatent) were estimated by the modified Ogur and Rosen technique described in the appendix, and the relative amounts of acid-soluble nucleotides and RNA present in the homogenate and fractions were calculated from the mean of the three values obtained. Additional aliquots of the supernatent were brought to pH 5 by the addition of 0.1 N. acetic acid in the cold, and the resulting white precipitate, which contained the pH 5 enzyme and any RNA associated with it, was separated from the pH 5 supernatent by centrifugation at 1500g. These two fractions were estimated for acid-soluble nucleotides and RNA in the same way.

Similar estimations were carried out on stationary cultures, obtained by inoculating flasks containing 750 mls. wort broth with 3 mls. stationary culture and incubating at 33°C for 40 hrs., and on

TABLE 5.

Distribution % of recovered RNA in cell fractions of S. pombe

State of Culture	pH 5 SN	pH 5 ppte.	SN	P	Ratio P/SN	Values for Debris omitted Mean P/Sn
Log phase	2.1	8.0	10.1	39.6	3.9	
	1.4	5.2	6.8	27.5	4.1	4.7 ± 1.2
	2.6	3.6	6.1	40.0	6.0	
Stationary	not determined		12.0	30.0	2.5	
			8.0	29.0	3.6	3.8 ± 0.9
	2.0	5.0	7.1	36.3	5.1	
	0.8	7.7.	10.5	41.6	4.0	
Lag phase	not determined		6.7	25.5	3.8	
			6.2	23.3	3.7	3.8

lag phase cultures, harvested one hour after the inoculation of 750 mls. fresh broth with the cells from a 750 ml. stationary culture.

Results.

There is no record of the presence of RNA in the cell walls of Baker's yeast (Korn and Northcote, 1960), and the trace of so-called RNA found in bacterial cell walls can probably be accounted for by contamination with other cell fractions (Mitchell and Moyle, 1951). As mentioned before, the presence of RNA in mitochondria is debatable. In the experiments of this series in which cell breakage counts were carried out, as far as can be judged from the approximate nature of the cell counts, all the RNA in the debris fraction could be accounted for by the number of unbroken cells present.* It can therefore be assumed that the results in Table 5 represent the distribution of cytoplasmic RNA in S.pombe.

Statistical analysis shows that there is no significant difference in the ratio of ribosomal:SRNA found in cultures of different ages, containing different proportions of cells which were actively growing and dividing. In all the cultures, approximately 80% of the RNA of the broken cells was recovered in the particle fraction, and the remainder in the supernatent. It should be borne in mind, however, that possible differences in the intracellular environment of cells at different stages in the life cycle, which may cause variation in the cellular organization of RNA, are eliminated when the cells are exposed to a new set of conditions which may upset

---

* Nuclear RNA would also presumably be present in the debris fraction, but the accuracy of these experiments was not such as to detect it.

TABLE 6

Distribution (%) of recovered acid-soluble nucleotides in cell fractions of *S. pombe*.

State of culture	SN	P	Debris
Log phase	76.4	12.7	12.7
	59.2	9.6	12.6
	67.0	9.1	14.0
Stationary	80.0	4.6	17.8
	64.0	1.9	2.9
	71.2	1.9	0
	81.8	2.7	4.6
Lag phase	70.5	7.8	0
	68.0	3.6	14.7

equilibria existing in the cell. Also slight differences in P/SN ratio between growing and stationary cultures would probably be masked at the level of accuracy achieved in these experiments. It would be more satisfactory to carry out similar experiments on synchronous cultures.

Of the SRNA, most was precipitated at pH 5 with the pH 5 enzyme.

The distribution of acid-soluble nucleotides (Table 6) in the living cell is difficult to assess from these experiments, since the nucleotides in the SN fraction included those which had diffused from the unbroken cells of the homogenate as well as those from the broken cells. (see p.79). The fact that cells treated as described on p.79 contained no acid-soluble nucleotides when fractionated suggests that those found in the particle and debris fractions were probably loosely adsorbed on to the particles and possibly on to the cell walls. From the point of view of later experiments, it should be noted that the amount of acid-soluble nucleotides found in the debris fraction never rose above 18% of the total in untreated cells.

The ratio of total RNA to total acid-soluble nucleotides in the homogenate (mean value from all experiments 2.7:1) is rather low compared with that obtained in the extraction experiments to be described later (mean value 3.9:1) and by Dr. P.D. Mitchell (3.4:1). This may mean that there is some degradation of RNA during cell breakage and fractionation.

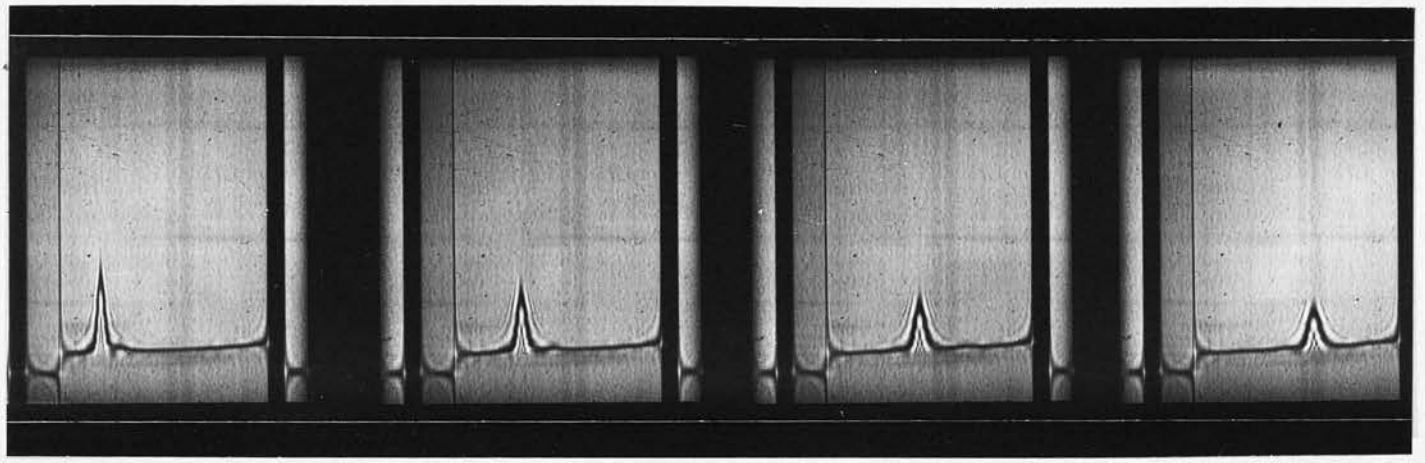


Fig.18. Sedimentation pattern of particles isolated from a log phase culture of S. pombe. Photographs at 15, 25, 35 and 45 minutes.

PROPERTIES OF THE PARTICULATE RNA IN S.POMBE.

Particles from stationary cultures were examined in a Spinco Model E analytical centrifuge at 20,410g (bar angle 45°) using Schleiren optics.* A single peak was observed from each preparation with a sedimentation rate varying from 72S to 86S (mean of seven determinations = 77.9S) (Fig. 18). The determination was repeated on a mixture of particles from growing and stationary cultures : the sedimentation pattern was the same as with particles from stationary cultures only.

Electron microscopy of sectioned S. pombe from growing cultures carried out by Mr. N. Maclean (unpublished) has demonstrated the presence of numerous particles free in the cytoplasm, ranging in size from approximately 100 A to approximately 200 A. Examination of the particle fraction isolated from a growing culture showed it to be composed of such particles.

---

* With the co-operation of Dr. C.T. Greenwood.

THE DIFFERENTIAL EXTRACTION OF RIBOSOMAL AND SOLUBLE RNA.

Any method for the separation of particulate from soluble RNA must be based on differences in their physical or chemical properties. The known differences between the two sorts of RNA which could possibly be used in the development of a differential extraction technique are firstly, the small molecular weight of SRNA as compared with particulate RNA, and secondly, the possible occurrence of SRNA unattached to protein as compared with the RNP nature of the ribosomes.

Early in the series of fractionation experiments, it was found that rapid freezing of intact cells by the immersion of the vessel containing them in a dry-ice and alcohol mixture (-75°C), followed by incubation at room temperature in ribosome buffer, resulted in the leakage from the cells of all the acid-soluble nucleotides. A similar affect of freezing on Saccharomyces cerevisiae has been reported by Hansen and Nossal, (1955) and on E. coli by B.P.Stephen (personal communication). Higuchi and Uemura (1959) obtained release of all the mononucleotides and also some oligo- and polynucleotides from S. cerevisiae with citrate buffer : they think it likely that the latter represent breakdown products of RNA accelerated by the chelation of magnesium ions by the citrate. In the present case it is thought that acid-soluble nucleotides only are released, as the ratio of material in the buffer extract absorbing at 260 mμ to the total RNA approximates to the 5:17 ratio for acid-soluble nucleotides : RNA obtained by Mitchell (in press). Also, lowering of the pH of the buffer extract to pH 5 by the addition of acetic acid did not cause the formation of a precipitate

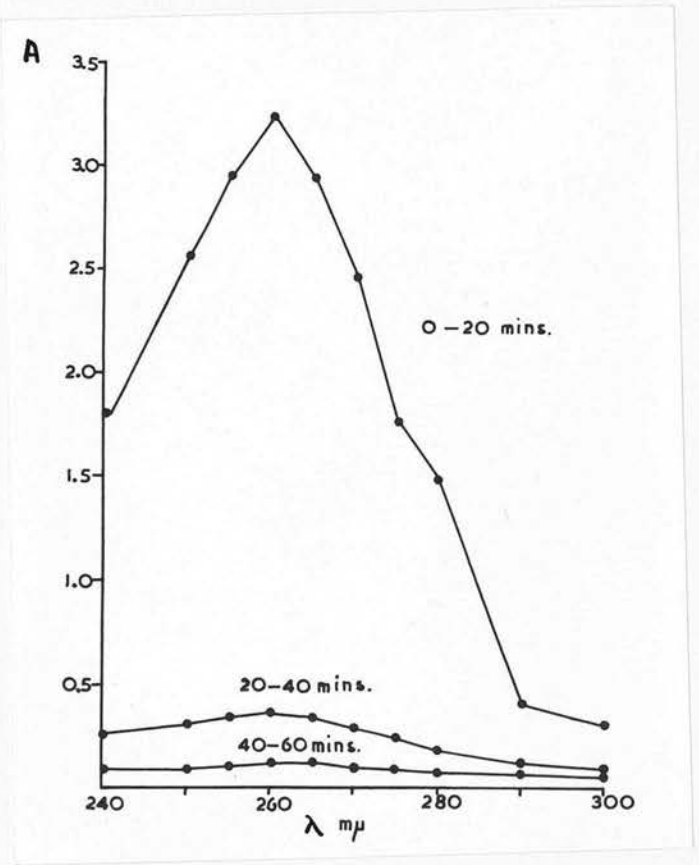


Fig.19. Absorption curves of ribosome buffer extracts of frozen and thawed S.pombe cells.

as would almost certainly have been the case had SRNA been extracted by the buffer. Fig. 19 shows that the extraction of the U.V. - absorbing material was virtually complete after one hour's incubation in ribosome buffer at room temperature. For routine extractions, the cells were frozen in about 10 mls. of buffer, allowed to thaw in about 30 mls. and to stand at room temperature for 90 mins. The cells were then spun down at 1500g and washed twice with buffer, the washings being added to the buffer extract.

Owing to this easy extraction of acid-soluble nucleotides from frozen cells, the problem of differentially extracting the SRNA would be solved if the latter could be broken down to its constituent mononucleotides (which could then diffuse from the cells) while leaving the ribosomes at least partially intact. Three possible methods of carrying this out were considered.

1. Acid hydrolysis.

For the purpose of these experiments, acid hydrolysis of RNA has two disadvantages. Firstly, complete hydrolysis of RNA with acid to its constituent purine bases and pyrimidine mononucleotides requires an inconveniently long time at the temperatures which can be used for cytochemical work; and secondly, it is known that the purine bases are released more quickly than the pyrimidine mononucleotides (Loring, 1955): it is therefore extremely likely that the purine bases of both the ribosomal and the SRNA would be completely removed before the liberation of the pyrimidine mononucleotides from either was accomplished. For these reasons, no attempt has been made here to extract differentially the two RNA fractions bythis method. However, recent work of Heyes (1960) is

of interest in this respect. He has found that the RNA of pea root meristems exists as two fractions differing in their extractibility with 1 N.PCA which possess different base rations. The relative amounts of the two fractions vary with the distance from the root tip, and Heyes suggests that one of the fractions (that most easily extracted with PCA) is associated with the microsomes, which are more concentrated at the tip than in older parts of the meristem, and that the other fraction is not attached to particulate protein. Until this speculation is placed on a firmer basis by the determination of the base ratios of the microsomal and SRNA, for example, his results are also open to the interpretation that the cellular RNA as a whole is changing in composition during growth and differentiation of the meristem.

2. Enzymic hydrolysis.

Early attempts to use ribonuclease in quantitative studies of RNA tended to be regarded with suspicion, owing to doubts about the specificity of the enzyme and to the fact that, in many cases, an undialysable 'core', consisting of a mixture of polynucleotides 3-5 residues in length (Markham and Smith, 1952), remained after exhaustive digestion. These difficulties have now been resolved; RNase is supplied as a crystalline non-proteolytic preparation, and in 1947, Greenstein et al. showed that complete degradation of RNA to dialysable breakdown products could be accomplished in the presence of a suitable electrolyte. This was confirmed by Markham and Smith (1952).

Tissières and Watson (1958) found that the degradation of pure

RNA with RNase always proceeded more rapidly than did that of the ribosomes of E. coli. Should all or part of the SRNA be unattached to protein, therefore, it would seem likely that it would be digested by the enzyme before the ribosomal RNA. Experiments by Brachet (1959) on living onion root tips showed that 40% of the SRNA was destroyed by RNase before the enzyme had any effect on the RNA of other cell fractions.

On these grounds, it was decided to proceed with experiments attempting the differential hydrolysis and extraction of the soluble and particulate RNA of frozen yeast cells with RNase.

Method

Frozen and thawed cells from log phase cultures were prepared as described above, and after removal of the acid-soluble nucleotides by buffer extraction, the suspension was divided into the number of equal aliquots necessary for the experiment (usually two, but sometimes four). Each aliquot was pipetted into a centrifuge tube and the final buffer washings were carried out to ensure the complete removal of the acid-soluble nucleotides. 5 mls. of a solution of crystalline RNase (Worthington), at a concentration of 0.1 mgm. per ml. of ribosome buffer (pH 6.9 at 37°C.) were added to the cells in the experimental tube(s) and 5 mls. of ribosome buffer were added to the control tube(s). The contents of all the tubes were well stirred and incubated in a water-bath at 37°C. for 16 mins. The cells were then spun down at room temperature for 4 mins. at 1500g, giving a total incubation period of 20 mins. The supernatents were removed to numbered McCartney bottles, and the cells were incubated

for a further 20 mins. with fresh medium. This process was repeated for 3-5 hours. The frequent renewal of the incubation medium ensured that enzyme exhaustion or inhibition did not occur. At the end of the experiment, 10 mls. cold 10% PCA was added to each of the tubes to extract any remaining RNA. The tubes were kept at $4^{o}C$ during this extraction and the PCA was replaced each day until no further U.V.-absorbing material could be detected in the extracts. The time necessary for complete extraction varied with the number of cells present and the amount of RNA remaining; in the case of control tubes containing approximately 1.5×10^{8} cells, U.V.-absorbing material continued to be extracted for about 100 hours, although approximately 90% was extracted in the first 18 hours.

The absorbance of each 20 min. extract and of the PCA extracts was measured at 260 mμ, and in addition, the absorbance of at least one extract from each tube was measured at 240, 280, and 300 mμ to ensure that the A_{260} was due to nucleotides. When the A_{260} was low, the A_{240} was always measured for the same reason.

The value of the A_{260} for each extract was multiplied by the volume of the extract, and the addition of all the products gave a measure of the total amount of RNA in the corresponding cell sample. The amounts in each extract could then be calculated in terms of percentage of the total. Although the RNase extracts differed from the PCA extracts in pH, no correction was applied for the variation in k_{260} which this introduces, as the alteration of the absorption curve by variation in pH is small where a mixture of nucleotides present in similar proportions is concerned. (Beavan et al., 1955).

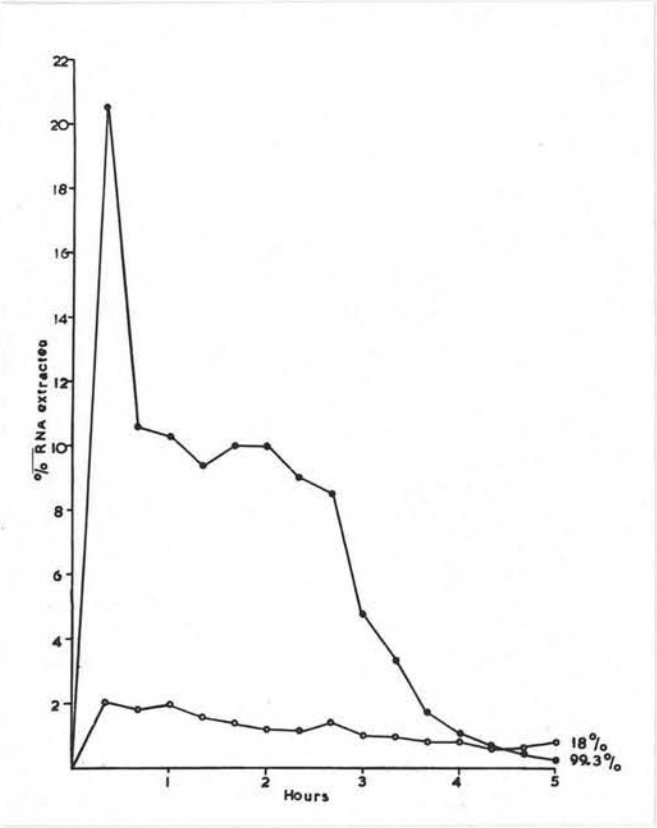


Fig.20. Course of extraction of RNA from frozen and thawed cells of S.pombe with RNase.

Solid circles — Cells incubated with 0.1 mgm./ml. RNase

Open circles — Controls.

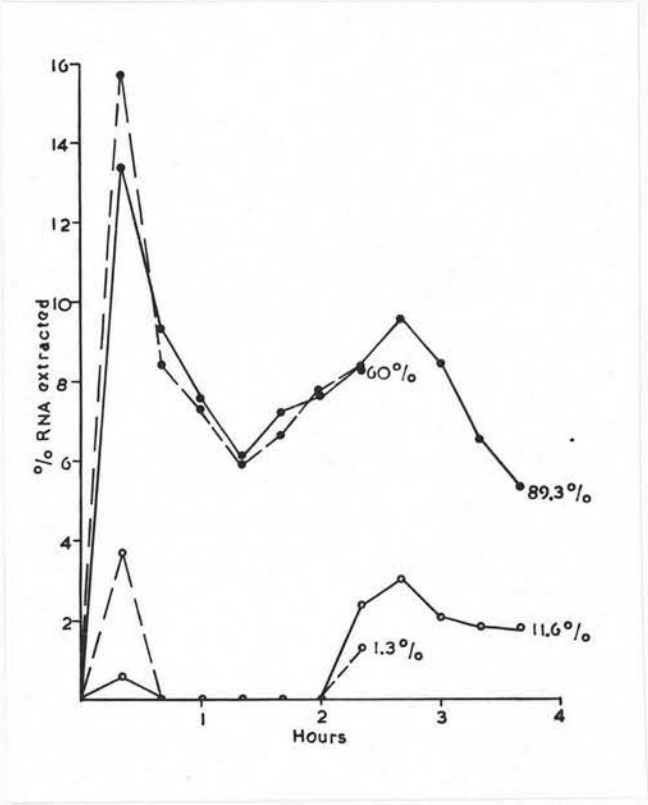


Fig.21. As Fig.20. Two extractions carried out simultaneously.

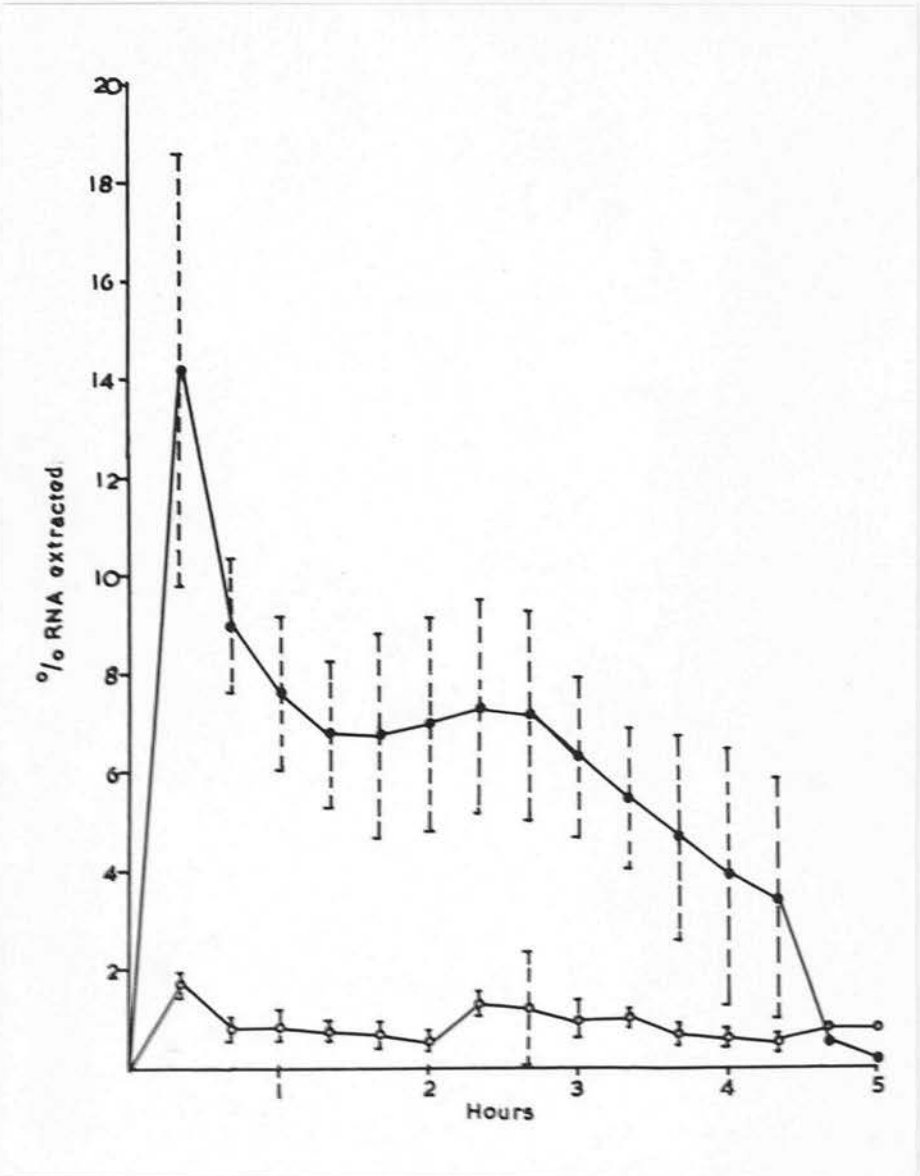


Fig.22. Curve drawn through the mean values obtained from five such experiments as those in Figs.20 and 21. Dotted lines denote standard deviations.

Results

The results of two typical experiments carried out under these conditions are presented graphically in Figs. 20 & 21. Three facts should be noted:

(a) The experiment in Fig. 20 shows that it is possible to extract the RNA completely with RNase from frozen and thawed, but ptherwise intact, cells.

(b) an amount ranging from 10-20% of the total RNA is extracted in the first 20 mins. under the conditions used. There is then a drop in the rate of extraction followed by a secondary rise and a slow fall which continues (sometimes rather irregularly) until extraction is complete. Although the relative heights of the two peaks and the time of the appearance of the second one varied from one experiment to another, the two phases of the extraction were always discernible. As the rate of action of RNase is to some extent dependent on substrate concentration (Schmidt, 1955), and the latter is falling throughout the experiment, the second rise may be of more significance than it appears from the curves. Fig. 22 is a curve drawn through the mean values obtained from five such experiments. There is no obvious reason for the large scatter between the values obtained at any one time from different experiments; it is possible that slight differences in the substrate concentration may play a part, as the numbers of cells used in the different experiments were not necessarily identical.

(c) there is a slight spontaneous breakdown of RNA in ribosome buffer alone, which may be due to the RNase of the cells themselves.

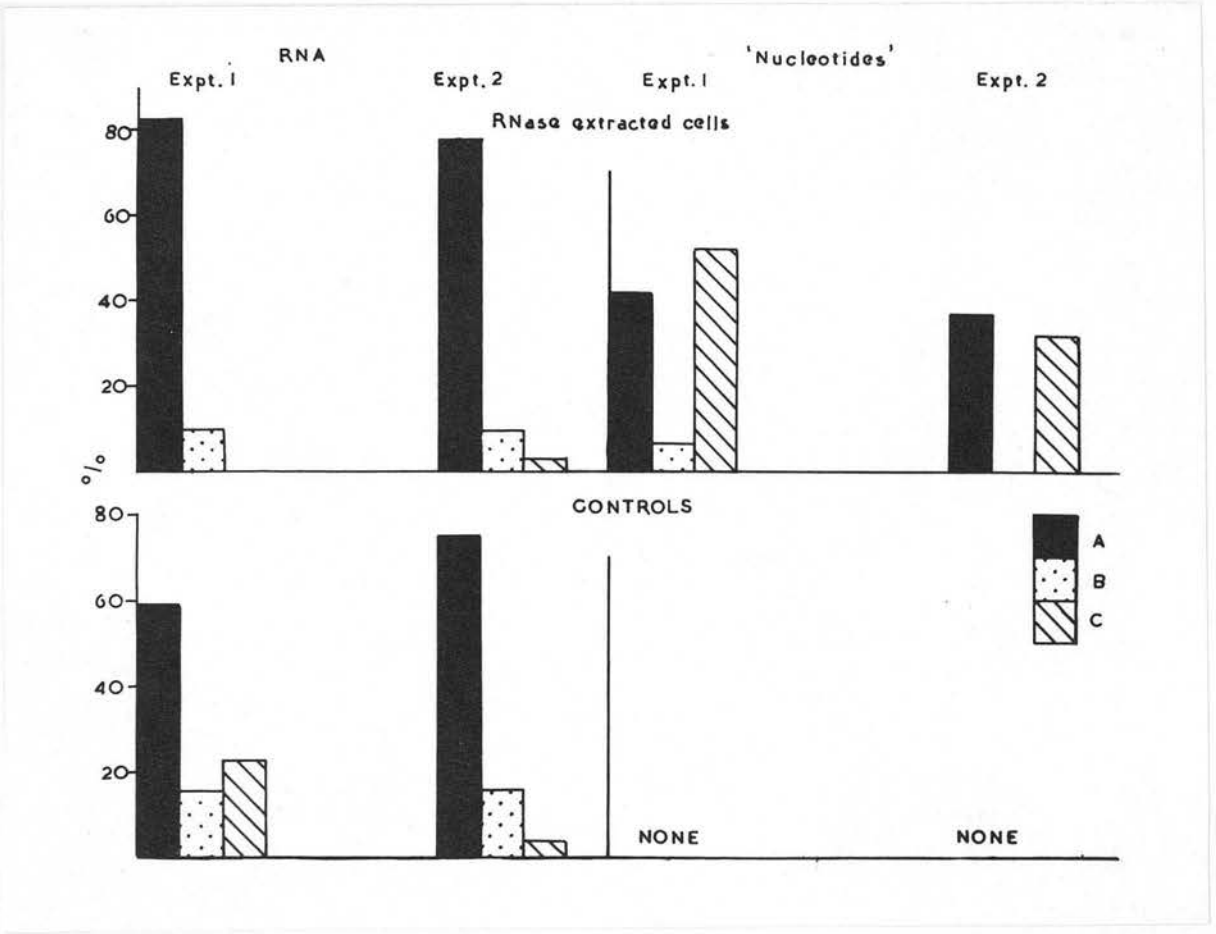


Fig.23. The distribution of recovered RNA and 'acid-soluble nucleotides' in cell fractions of S.pombe partially extracted with RNase. A. Debris fraction. B. Particle fraction. C. Supernatent fraction.

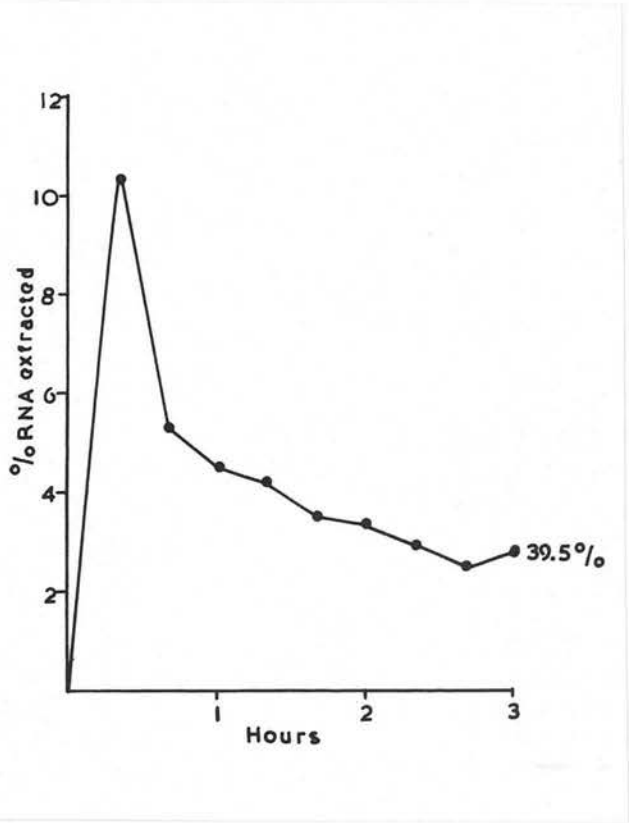


Fig. 24. Course of RNA extraction from S. pombe with a reduced concentration of RNase (10 µgm./ml.)

This is sometimes a little larger in the first 20 min. period than in the following ones; this may be due to a small amount of acid-soluble nucleotides remaining in the cells after the buffer extraction, or to the slight lag (never more than a minute) between the last wash in ribosomes buffer and incubation at 37°C. The spontaneous breakdown sometimes tends to rise towards the end of the experiment, and sometimes parallels the enzyme extraction curve, but if the values due to this are subtracted from those obtained by RNase extraction, the two-phase nature of the latter curve still remains.*

The shape of the extraction curve can be interpreted as representing the summation of two overlapping curves, the first produced by a quick reaction involving about 20% of the total RNA, the second representing a reaction which is less sensitive to RNase, slower in reaching its maximum and slower to die away, and which involves the remainder of the RNA. Assuming that this interpretation is correct, from the point of view of these experiments, one would hope that the first and second reactions represent the extraction of the soluble and the particulate RNA respectively, but the possibility that the nature and action of the enzyme itself may play a part in producing the biphasic curve must be considered. Certain internucleotide bonds only are split by the enzyme, so that even after exhaustive digestion the end-products consist of 3' uridylic

---

* Reduction of the concentration of RNase appears partially to suppress, or at least delay, the secondary rise. Fig.24 represents the course of extraction in an experiment in which the enzyme concentration was reduced to one-tenth its value in the former experiments i.e. to 10ug. per ml. buffer)

acid, 3' cytidylic acid, and a considerable number of oligonucleotides in varying degrees of polymerisation, (Schmidt, 1955). As has been shown, it is possible to obtain complete extraction of these breakdown products from frozen yeasts, but it may be that the pyrimidine nucleotides are able to pass out of the cell into the extraction medium more quickly than the oligonucleotides, and that the biphasic curve is due to this differential extraction. In this case, it would be expected that the A_{280} : A_{260} and the A_{240} : A_{260} ratios of the two fractions might differ, but no significant differences were found. Investigation of the base ratios of the two fractions would confirm this point. Another complicating factor is the possible heterogeneity of the enzyme. Hakim (1957) found that Worthington RNase consisted of two components which tended to attack different linkages preferentially. If this is the explanation of the biphasic curve, it would also tend to result in different base ratios in the two fractions.

In spite of possible complications introduced by the enzyme, the relative amounts of material extracted during the first hour and during the remainder of the experiment (approximately 20% and 80% respectively) seemed to support the hypothesis that SRNA was preferentially degraded by RNase and extracted from the cell before the particular RNA. To investigate this possibility, two fractionation experiments were carried out.

After three 20 min. extraction periods in RNase solution (10ug. per ml. ribosome buffer) and ribosome buffer respectively, the experimental and control cells were refrozen and stored at -14°C. overnight. They were then thawed and transferred to the cold Hughes

press as a thick suspension, and broken and fractionated, and the acid-soluble nucleotides and RNA were estimated and described as above. The results are presented in Fig. 23.

The high proportion of RNA present in the debris fraction of the control cells is associated with the rather low percentage of cells broken; the amounts of RNA in the particle and SN fractions were therefore small and the accuracy of the measurements is probably not very high. This may help to account for the equivocal nature of the results. From Expt. 1, it appears that the RNase treatment had indeed broken down all the SRNA, and that it resulted in a considerable amount of partially degraded RNA, which, although it was extractable from the treated cells with 2% PCA and could therefore be defined as acid-soluble mucleotides, did not leak from the cells into the extraction medium. The amount of this material present was half as great as the amount of the RNA recovered, suggesting that some of the particulate RNA had also been broken down during the first hour. Such material was completely absent from the control cells. The proportion of particulate to soluble RNA in the latter was quite different from that found in previous fractionation experiments, and it was thought that perhaps the previous removal of the nucleotides may have upset an equilibrium between the two types of RNA, resulting in a breakdown of particulate RNA to form SRNA. This idea was not confirmed by the second experiment, where the proportion of particulate RNA in the control cells was if anything higher than usual. The RNase treatment in this experiment again resulted in an accumulation of breakdown products, but in this case, these amounted to 1.5 times the recovered RNA and must have resulted from the breakdown of both

particulate and SRNA, as these were in approximately the proportions found in untreated cells. In the second experiment, it was noticed that the SRNA of the RNase treated cells was present, not in the pH 5 precipitate, as it is in the untreated cells, but in the pH 5 SN. This agrees with an experimental finding of van der Decken and Hultin (1957), namely that treatment with RNase renders the RNA component of the pH 5 enzyme less precipitable.

The presence of the partially degraded RNA shows that particulate RNA is being broken down by the enzyme during the first hour of incubation, although not a great deal of it may be sufficiently degraded to leak from the cells. It would be more satisfactory if either the action of the enzyme, or the nature of the substrate, could be modified so that only one type of RNA (either the particulate or the soluble) were attacked. This was attempted in two ways:

(i) The effect of mercuric chloride

In experiments with frozen-substituted rat liver, Bell (1959) found that the inclusion of mercuric chloride in the substituting solvent modified the action of RNase on the fixed sections. Material fixed in methanol containing 1% $HgCl_2$ still stained with pyronine after 1 hour's treatment with RNase (although not so densely as control sections untreated with RNase), while sections from material fixed in methanol alone failed to stain with pyronine after incubation with the enzyme for 30 mins. It appears therefore that the mercuric chloride protected part of the cytoplasmic RNA from RNase action, so it was decided to investigate the affect of $HgCl_2$ on the RNase extraction technique described above.

Method

The cells from two 750 ml. log phase cultures were pooled and washed with ribosome buffer. They were frozen and thawed, and the acid-soluble nucleotides were extracted as usual, the suspension being divided into four equal aliquots. After two routine washes with ribosome buffer, 10 mls. of buffer were added to aliquots 1 and 2, and 10 mls. of the same buffer containing 1% $HgCl_2$ were added to aliquots 3 and 4. All four were left at room temperature for an hour and then washed four times with ribosome buffer. Measurement of the absorption of the washings from aliquots 1 and 2 showed that a negligible amount of U.V. absorbing material had been removed in this hour by the buffer, while absorption measurements of the final washing from tubes 3 and 4 showed that very little $HgCl_2$ was present in it and that it had no absorption at 260 mu. Aliquots 1 and 3 were then incubated with 0.1 mgm. per ml. RNase in ribosome buffer, while aliquots 2 and 4 were incubated in buffer alone. RNase treatment was carried out as usual for a total time of 3 hrs., the RNA remaining in the cells being extracted with 10% PCA at 4°C as described above.

Results and Conclusion

The mercuric chloride prevented completely the extraction of RNA with RNase and also modified its extraction with 10% PCA. After incubation for 3 hrs., during which no U.V.- absorbing material was extracted from the cells which had been treated with $HgCl_2$, 10% PCA was added to all four aliquots. U.V.-absorbing material was completely removed from the cells untreated with $HgCl_2$ in 95 hours,

while it continued to be extracted from the treated cells for 184 hours. This fact, together with Zittle's (1946) finding that $HgCl_2$ is not an inhibitor of RNase (although the concentrations he used were considerably lower than those used here), makes it seem probable that the affect of the salt is on the substrate rather than on the enzyme. Alternatively, the permeability of the cell wall may have been altered, so that it was impermeable either to the enzyme or to the breakdown products of its activity, and less permeable than untreated cells either to PCA or to the products of PCA hydrolysis. The difference between these results and those of Bell may be due to the fact that he was using fixed material, in which the protein would already be denatured.

(ii) The effect of magnesium concentration

As has been mentioned before, the physical properties of ribosomes are dependent on the magnesium concentration of the medium. Many workers have found that the RNP particles tend to dissociate into smaller components in low Mg^{++} concentrations, while Chao (1957) found that ribosomes prepared from Saccharomyces cerevisiae were precipitated by bivalent ions, at a concentration of 0.01 M. Shigeura and Chargaff (1960) have recently demonstrated that the action of RNase on RNP also varies with the concentration of Mg^{++} present, concentrations down to 0.001 M. (which is the concentration used in the above experiments) having considerable inhibitory activity on the enzyme. The action on RNA free from protein, on the other hand, was not affected by the magnesium concentration. They consider that a gradual dissociation of RNP is a prerequisite for RNase action, and that Mg^{++} ions

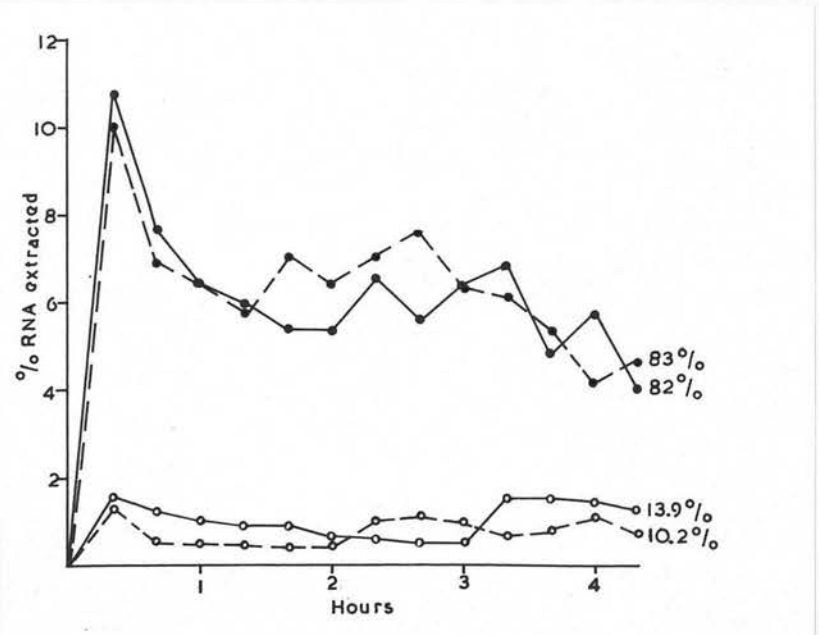


Fig.25. Extraction of RNA from frozen and thawed cells of S.pombe, with RNase, showing the effect of varying concentrations of magnesium.

Solid circles, continuous line	0.001 M. Mg^{++}, 0.1 mgm./ml. RNase.
Solid circles, broken line	0.01 M. Mg^{++}, 0.1 mgm./ml. RNase.
Open circles, continuous line	0.001 M. Mg^{++}, control
Open circles, broken line	0.01 M. Mg^{++}, control

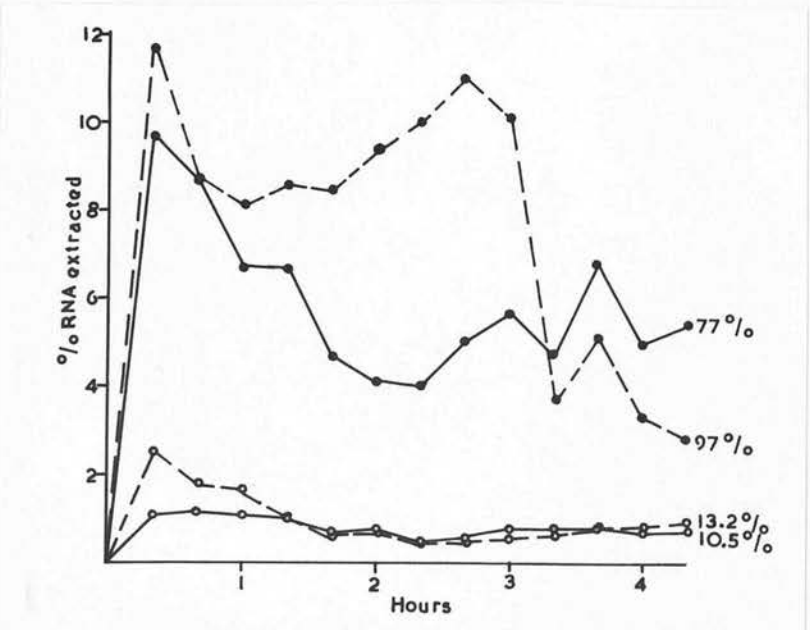


Fig.26. Extraction of RNA from frozen and thawed cells of S.pombe with RNase, showing the effect of varying concentrations of magnesium.

Solid circles, continuous line	0.001 M. Mg^{++},	0.1 mgm./ml. RNase.
Solid circles, broken line	without Mg^{++},	0.1mgm./ml. RNase.
Open circles, continuous line	0.001 M Mg^{++},	control
Open circles, broken line	without Mg^{++},	control

stabilise the conjugated protein. If all or part of the SRNA were present free from protein, therefore, it should be possible to inhibit the breakdown of the ribosomes without affecting that of the SRNA, by raising the concentration of magnesium.

Method.

Four aliquots of cells were prepared as for the usual RNase extraction and were incubated as follows:

i) with 0.1 mgm. per ml. RNase in ribosome buffer i.e. containing 0.001 M. Mg^{++}

ii) with ribosome buffer alone

iii) with 0.1 mgm. per ml. RNase in buffer with the concentration of Mg^{++} raised to 0.01 M.

iv) with this buffer alone.

The extractions were carried out in the usual way.

In a second experiment, four aliquots were incubated as follows:

(i) and (ii) as (i) and (ii) above.

(iii) with 0.1 mgm. per ml. RNase in buffer without Mg^{++}

(iv) with this buffer alone.

Results and Conclusions

The results of these two experiments are presented in Figs. 25 and 26. It is obvious that raising the Mg^{++} tenfold had no significant effect on the course of the extraction. Omitting magnesium from the extraction medium increased the rate of extraction, especially as regards the second phase. There is also a slight increase in extraction rate at the beginning of the curve which also appears in the control curve. From these results it seems that

RNase action in this system is partially inhibited by a range of Mg^{++} concentrations from 0.001 M to 0.01 M., but that this inhibition only slightly affects the form of the first part of the curve. This suggests that most of the SRNA occurs free from protein, as Tissières found to be the case in E. coli, and supports the hypothesis that the secondary rise in the extraction curves is due to the breakdown of RNP. Unfortunately the discontinuity of the breakdown of the two types of RNA is not sufficiently great to form a basis for a differential extraction technique, so this approach was abandoned and the effects of another form of hydrolysis were investigated.

3. Alkaline hydrolysis.

Mild alkaline hydrolysis is an established method of effecting the quantitative hydrolysis of RNA to its constituent nucleotides (Davidson, 1953); Loring, 1955). Provided that no complicating side-affects occur, the action of alkali on the RNA of the intact cell should be easier to follow with an extraction technique than was the course of RNase digestion, where the formation of partially degraded RNA as an intermediate breakdown product which could not pass through the cell wall resulted in a lag between the RNA breakdown and its detection. The effect of alkali on the RNP particles of the cell seems to vary. Chao (1957) found that the 80S particles from S.cerevisiae were completely dissociated into smaller particles at pH 9.7 and that prolonged dialysis at pH 10 caused aggregations of protein to form leaving all the nucleic acid in solution. T'so, (1958), working on ribosomes from pea seedlings, obtained dissociations of the 80S particles into smaller units at pH values above 6.5. Gilchrist and

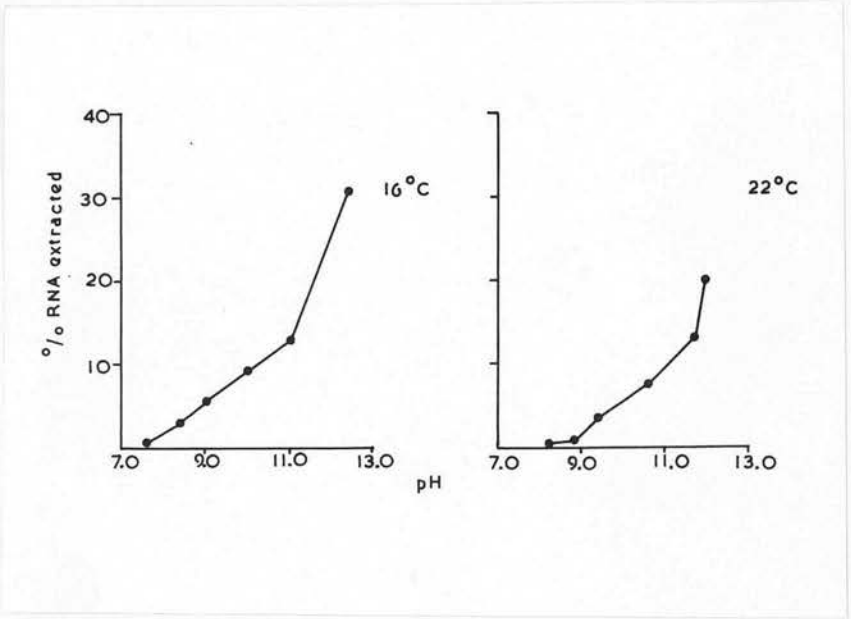


Fig.27. The extraction of RNA from frozen and thawed cells of S.pombe with alkaline buffers. Cumulative curves.

Bock (1958) on the other hand, found that the 80S particles from Azotobacter vinelandii were precipitated at pH values above 7.5. It was thought that it might be of interest to investigate the effect of high pH on the RNA of S. pombe.

Method

Frozen and thawed cells from growing cultures were prepared, and the acid-soluble nucleotides were removed as before, the cell suspension being divided into the requisite number of aliquots. Two aliquots were used for each of two preliminary experiments, one aliquot in each being incubated with ribosome buffer (pH 7.2 at 22°C.) The other aliquots were incubated for periods of one hour in 5 ml. aliquots of Sorensen borate-NaOH (-HCl) buffer (Hale, 1958) of increasing pH. After the final incubation (at about pH 12.0), each aliquot was washed twice with ribosome buffer, the washings being pooled and kept for spectrophotometric measurement. Cold 10% PCA was then added to the cells to extract the remaining RNA. The U.V. absorption of all the extracts was measured as in previous experiments, and the relative amounts of RNA in each were calculated.

The results are presented in Fig. 27. Unlike the extraction curves drawn for the course of RNase extraction, here the course of the extraction is plotted as a cumulative curve. There is a fairly steady extraction of U.V. -absorbing material (presumably mononucleotides) for the first five hours, during which the pH was increased from 7.6 to between 11.0 and 12.0. At pH 12.0, however, there is a inflection in the curve, indicating that the rate of RNA breakdown is sharply increased at this pH. To confirm that this result was

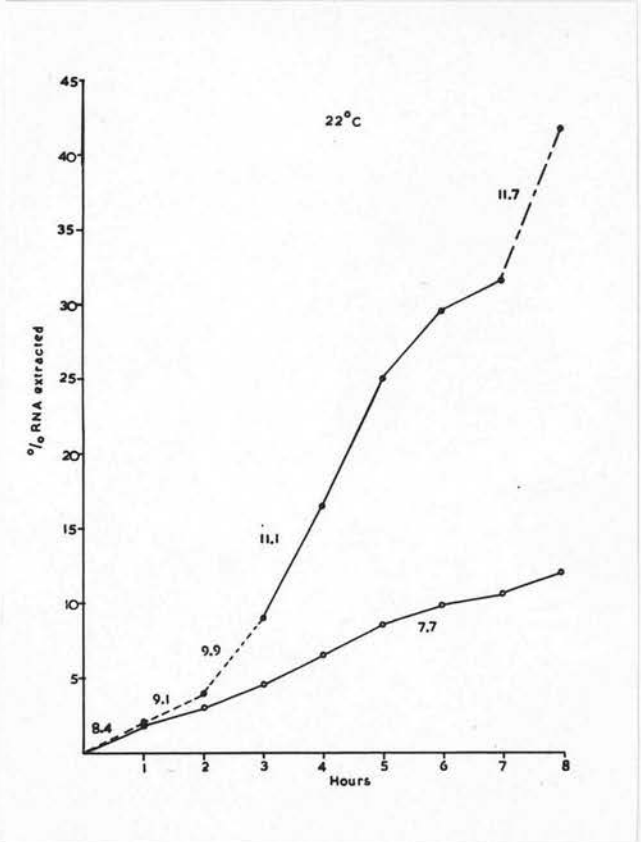


Fig.28. The extraction of RNA from frozen and thawed cells of S.pombe with alkaline buffers. Cumulative curves. The figures refer to pH values.

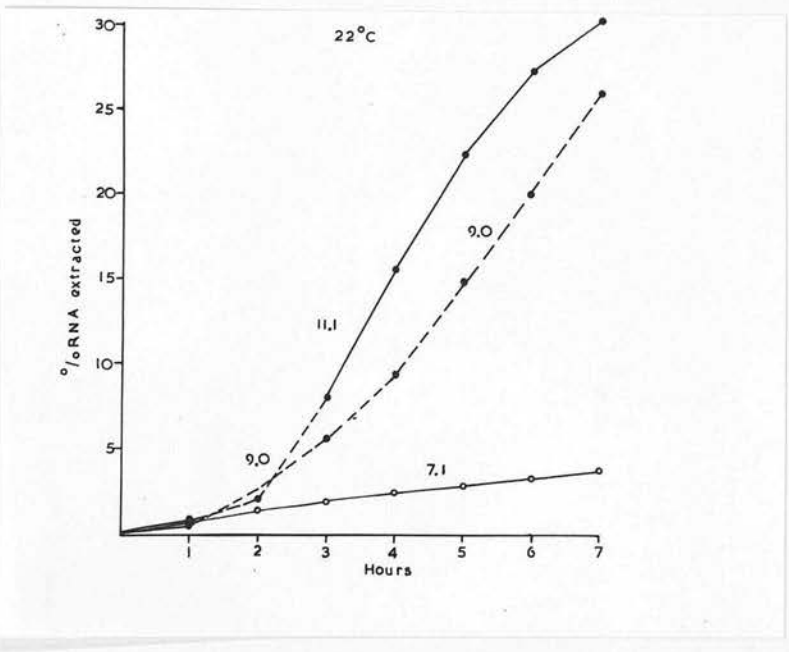


Fig.29. As 28.

due to the increased pH, rather than merely to the effect of prolonged incubation, the experiment was repeated, the incubation at pH 11.1 being continued for 4 hours before the pH was again increased, with the result shown in Fig.28[*] In this case, borate-NaOH(+HCl) buffer at pH 7.7 was used for the control incubation, instead of ribosome buffer. This experiment shows clearly that time of incubation alone is not an important factor in the increased rate of extraction at very high pH. Continued incubation at pH 11.1 resulted in a fall in the rate of RNA extraction. When the pH was raised to 11.7, there was again a sharp rise in the extraction rate. This result could be brought about in two ways. Firstly, it may be that at each pH a certain limited amount of RNA is extractable; or secondly, it is possible that at all pH's up to about 11.5, a certain fraction, representing 30-35% of the total RNA is extractable. At pH 11.1, the breakdown and extraction of this fraction takes 7 hours; at a lower pH, it may take longer. The result of the next experiment (Fig.29), in which an additional aliquot of the cell suspension was incubated at pH 9.0, supports the latter possibility. The rate of extraction at pH 9.0 continued undiminished after 7 hours incubation, while the rate at 11.1 behaved as in the previous experiment. It seems likely, therefore, that 30-35% of the RNA of the cells was more susceptible to alkaline hydrolysis below pH 11.1 than the remainder.

---

[*] If the incubation at pH 11.1 was not preceded by treatment at lower pH values, no more RNA was extracted from the experimental cells than from the controls. In the light of fractionation experiments described later, it seems likely that at high pH, both the ribosomes and the SRNA are rapidly aggregated, most of the RNA thus being protected from the hydrolytic action of the alkali.

The next step was to fractionate the cells thus treated and to determine which fraction of the RNA had been broken down. It was possible that certain bonds in all the RNA of the cells were being attacked, resulting in the breakdown of part of the RNA in both the ribosomal and the soluble fractions, but this hypothesis is not supported by the $A_{280}:A_{260}$ and $A_{240}:A_{260}$ ratios of the extracts, which approximate to those calculated from the absorption curve of a mixture of nucleotides in similar proportions to those found in polymerised RNA (Beavan et al., 1955). As with the RNase extraction, investigation of the base ratios of the two RNA fractions would clarify this point. The amount of RNA degraded up to pH 11.1 was sufficiently near the amount of SRNA present in the cell for the possibility to remain that differential extraction had been achieved.

Two fractionation experiments were carried out. After incubation in the alkaline buffers for 8 hours as described, the cells were washed twice with ribosome buffer, and the experimental and control aliquots (which contained a known number of cells), were frozen in a dry-ice and alcohol mixture and stored at -14°C. The cells were then broken as usual and cell counts were carried out to determine the number both the number of cells present in the homogenate and the percentage of broken cells. In this way it was possible to estimate the amount of RNA which had been extracted during incubation from the broken cells in the homogenate. Fractionations and estimation of the acid-soluble nucleotides and the RNA in the fractions werecarried out as previously described.

Results and Conclusion

The histograms in Fig.30 refer to the RNA distribution in broken

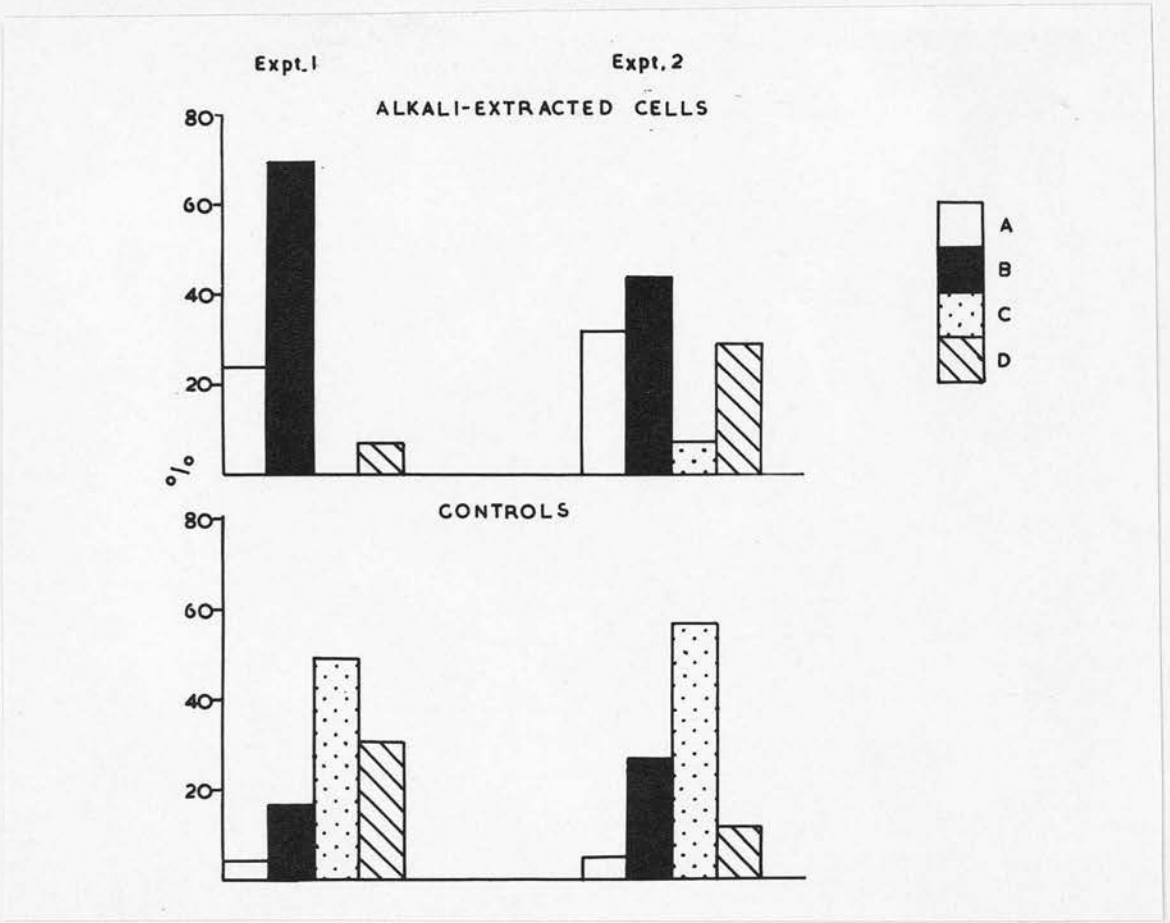


Fig.30. Distribution of recovered RNA in alkaline extracts and cell fractions of frozen, broken and fractionated cells of S.pombe. A.Alkaline extract. B.Debris fraction.C.Particle fraction. D.Supernatent fraction.

cells only. Although the results of the two experiments do not completely agree they have several points in common with one another. No U.V.- absorbing material was detected in the 2% PCA extracts from any of the fractions, in contrast to those obtained after RNase extraction. This is what would be expected from the difference in the mode of action and specificity of action of alkali and RNase on the bonds present in polymerised RNA. In both the experimental and the control cells, there was RNA present in the debris of the broken cells. In the experimental cells of the first experiment, the amount in the debris is as high as 70% of the total RNA present before extraction. For the reason given above (p. 75) this is not thought to be due to the presence of RNA in cell walls and mitochondria, but to the affect of the borate buffer. The rise in debris RNA is accompanied by a fall in ribosomal RNA, which is particularly marked in the experimental cells, and which cannot be wholly accounted for by the amount of RNA extracted during incubation in either the experimental or the control cells. The effect of the extraction in alkaline buffers of the SRNA is variable and does not seem to bear any relation to the amount of RNA extracted in the second experiment, but in the first it may not be wholly coincidental that the sums of the amounts of the extracted RNA and SRNA are almost the same in both the experimental and the control cells, as are the sums of the debris and the particle RNA (Table 7).

TABLE 7.

	Extracted RNA	SRNA	Sum
Experimental	23.5	7.0	30.5
Control	4.0	30.1	34.1

	Debris RNA	Ribosomal RNA	Sum
Experimental	69.5	0	69.5
Control	16.1	49.6	65.7

This fact, together with the large amount of RNA found in the debris fraction and the small amount in the ribosomes, indicates that the latter aggregated irreversibly in the alkaline buffer of pH 11.1 and were thus sedimented with the debris fraction. This occurred to a certain extent even in the controls. The ribosomes of S.pombe in alkali thus seem to behave more like those of Azotobacter vinelandii, studied by Gilchreist and Bock (1958), than /like/ those of Saccharomyces cerevisiae (Chao, 1957). The result of the second experiment suggests that some breakdown of particulate RNA does occur even in preference to SRNA, although an alternative explanation may be that during the extraction of SRNA, partially depolymerised RNA is being released from the ribosomes, and appears in the supernatent fraction of the broken cells. It is known that dilute alkali can be used to separate RNA from protein (Magasanik, 1955). In either case, alkaline hydrolysis by the buffer used here would seem to be less satisfactory than enzymic hydrolysis as a method for the differential breakdown of particulate and soluble RNA. It is possible that other buffers may have a different action, and that one could be found which would aggregate all the particulate RNA and extract all the soluble acid RNA.

CONCLUSION TO PART II

Considered as a whole, the results in Part II show that the cellular organisation of RNA in Schizosaccharomyces pombe is similar to that in other micro-organisms which have been studied. Eighty per cent of the cytoplasmic RNA is in the form of particles with a uniform sedimentation constant of approximately 80S; the remainder is found in the supernatent fraction. From the simplicity of the fractionation procedure, it seemed likely that the particles occurred free in the cytoplasm, and not attached to any form of endoplasmic reticulum. This was confirmed by the electron microscope study mentioned. The organisation of the particles is perhaps simpler than, for instance, in Saccharomyces cerevisiae, where the sedimentation pattern varies with the physiological state of the culture (Ashikawa, 1958). Here, there was no evidence that the proportion of ribosomal : SRNA changes during the life cycle of the cell, nor that particles of other sizes appear during growth of the cells.

The methods used in the attempt to extract differentially the soluble and particulate RNA were not completely successful. The use of RNase is perhaps the more promising of the two, provided that the conditions can be adjusted to exaggerate the discontinuity of the reaction, preferably by eliminating the second part of it, and that the procedure can be sufficiently standardised.

In spite of the failure to accomplish this aim, the experiments throw a little more light on the nature of the RNA of S.pombe. From the results of the RNase action in varying concentrations of magnesium, it seems likely (if the action of magnesium

within the cells is similar to that found by Shigeura and Chargaff on RNA and RNP *in vitro*) that most of the SRNA is free from protein, and that it is more susceptible to the action of RNase than is the remainder of the RNA. Further experiments are needed to investigate the possibility that the SRNA is that fraction (or at least part of that fraction) of the cellular RNA removed by freeze-substitution followed by treatment with a lipid solvent, as described in the U.V. microspectrophotometric experiments in Part I.

The action of the alkaline buffers used appears to be two-fold: 30-35% of the total RNA was hydrolysed and extracted by buffers between pH7 and pH11.5, while most of the remaining RNA was precipitated or was adsorbed on to the debris fraction and was sedimented with it. Unfortunately the fractions which reacted in these two ways did not correspond to the SRNA and the particulate RNA. Again, it is possible that the extracted RNA corresponded to the fraction lost from the cells during freeze-substitution and lipid extraction mentioned in Part I, and further experiments are needed to confirm this.

SUMMARY

PART I.

I. The need for quantitative cytochemical techniques for the investigation of cellular metabolism is discussed, and the importance of the comparison of results obtained by different techniques, and with those obtained by biochemical techniques, is emphasised.

2. The quantitative cytochemical methods available for the estimation of RNA in individual cells are described and their respective merits are discussed.

Results obtained by ultra-violet microspectrophotometry and by visible light microphotometry after Azure B staining of Schizosaccharomyces pombe cells are described and the following conclusions are drawn:

i) In unfixed cells the amount of RNA per cell increases from approximately 2.5μμg. to approximately 5.0μμg. during the life cycle: this is in good agreement with results obtained by a biochemical method. From the curve obtained it is suggested that the rate of RNA synthesis increases slightly throughout the life cycle. It is suggested that some of the RNA is non-functional as regards the production of protein.

ii) The amount of RNA in frozen-substituted cells which have been treated with a lipid solvent is considerably smaller, and it is suggested that this is due to the extraction of part of the RNA.

iii) From comparison of these results with those obtained by microphotometry in visible light after Azure B staining it is concluded that U.V. microspectrophotometry is a more satisfactory method for these cells.

iv) Comparison of the RNA synthesis curves obtained by measurements of Azure B intensity in cells grown at 17°C. and 33°C. suggests that the decrease in temperature slows down the mechanism responsible for cell division more than the mechanisms responsible for protein and RNA synthesis.

3. From results obtained by U.V. microspectrophotometry of RNase extracts of interphase mouse fibroblasts grown in tissue culture, it is suggested that the RNA content may increase during interphase to more than twice the amount present after division.

PART II.

4. The heterogeneity of the RNA within the cell is described, together with cytochemical methods available for the quantitative investigation of the fractions.

5. The organisation of cytoplasmic RNA in general, and that of S.pombe in particular, is described. Eighty per cent of the RNA appears to be present in particles (ribosomes) similar to those in other micro-organisms, the remainder representing the 'soluble' RNA.

6. Attempts to extract the soluble RNA from intact cells while leaving the ribosomal RNA intact, were not wholly successful, but it is felt that enzymic hydrolysis, combined with an appropriate concentration of magnesium, is the most promising approach. From

the results obtained it is suggested that most of the SRNA occurs free from protein.

APPENDIX

I DETERMINATION OF THE BASE RATIO OF THE RNA OF SCHIZOSACCHAROMYCES POMBE

This was carried out by the method of Osawa et al. (1958) and involved quantitative hydrolysis of the RNA to mononucleotides followed by ion exchange chromatography.

The washed cells from two 750 ml. log phase cultures were treated with 2% PCA at 4°C to remove the acid-soluble nucleotides. Two 10 min. in extractions with 20 mls. PCA were used. The cells were then treated with 95% ethanol (three extractions of 20 mins. at room temperature) and ether (two extractions of 10 mins. at 40°C.) to remove the lipid, and were then air-dried in a weighing bottle of known weight. The resulting white powder was weighed, 0.5 N. KOH was added (1 ml. for each 100mgm. of powder), and the suspension was incubated at 37°C. for 20 hours. It was acidified with 60% PCA in the cold to precipitate proteins and DNA, and after centrifugation, the supernatent was neutralised to pH 7-8 with 6 N. KOH in the cold to precipitate the potassium chlorate. The supernatent containing the mononucleotide mixture was removed after centrifugation and was stored at 4°C. The volume of the supernatent was 3-4 mls. and looked slightly turbid owing to the very high concentration of mononucleotides. A very small sample (0.1 ml.) was diluted 100 times and its absorption at 240, 260, 280 and 300 mμ was determined.

The ion- exchange system is adapted for small quantities of nucleotides from the 'formic acid system' of Hurlbert et al. (1954). Dowex-1 (cross-linkage 2%, 200-400 mesh) was washed as an aqueous slurry into a column 0.5 cm. in diameter and 20 cm. long, and was packed tightly by drawing off excess water with negative air-pressure

exerted with a filter pump, which was used for all washing procedures. The resin was converted into the formate form by washing it first with 50 mls. of a mixture of equal quantities of 6 N. formic acid and 1 M. sodium formate, and secondly with 50 mls. of 88% formic acid. It was then washed with distilled water, until the effluent was at neutral pH i.e. when all the excess formic acid had been removed. 1.5-2.0 mls. of the nucleotide mixture were then pipetted on to the top of the column, and were drawn into it by applying negative air pressure to the base. The adsorbed nucleotides were eluted with formic acid of continuously increasing concentration. A 50 ml. mixing flask containing a magnetic stirrer was connected to the column by polythene tubing containing distilled water. 30 mls. 1 N. formic acid were added to the flask, which was supplied by a reservoir containing 200 mls. 4 N. formic acid. A slight head of water pressure was necessary to force the eluent through the system at the required rate, and was adjusted to deliver 35 drops (about 3 mls.) every 15 mins. The 35-drop samples were collected automatically, about 70 samples being sufficient for the complete elution of all the nucleotides.

The absorption of each sample was measured at 260 and 280 mμ, and then the contents of all the tubes in each peak were pooled, the volume measured, and the absorptions at these wavelengths measured again. The amount of each nucleotide was then calculated, using the mM extinction coefficients given by Osawa et al. (1958), i.e.:

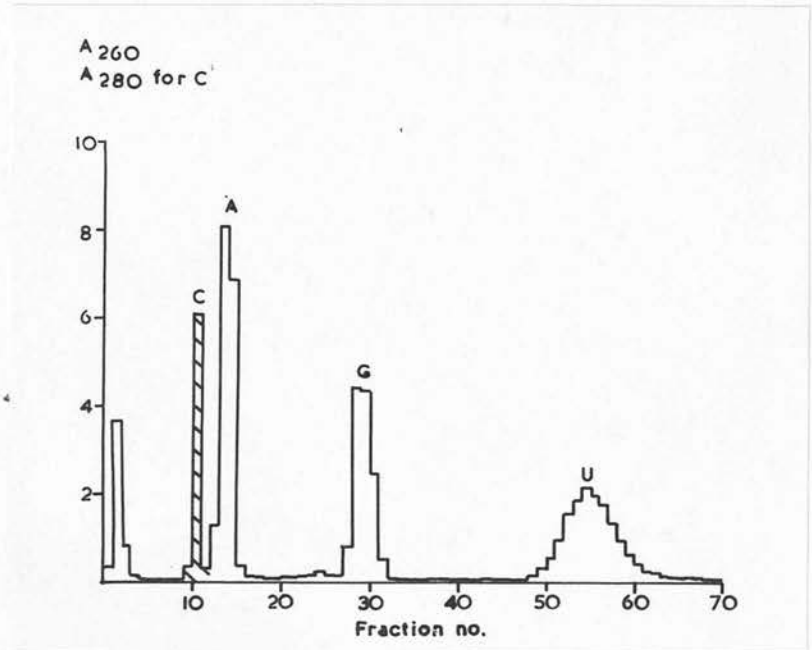


Fig.31. Elution diagram of mononucleotides from hydrolysed RNA of S.pombe.
A adenylic acid C cytidylic acid
G guanylic acid U uridylic acid
Cross-hatching denoted that the absorption measurements of he fractions concerned were carried out at 280 mμ

adenylic acid	14.2 at 260mμ
guanylic acid	11.8 at 260mμ
cytidylic acid	13.0 at 280mμ
uridylic acid	9.9 at 260mμ

Results

A typical elution diagram is shown in Fig. 31. The identification of each peak was carried out by the determination of the $A_{260}:A_{280}$ ratio. For the four main peaks, allowing for the slight differences in pH, these corresponded with those given in Beavan et al. (1955) for cytidylic, adenylic, guanylic and uridylic acids in that order. There was a small initial peak of unindentified material accounting for approximately 3.5% of the total absorption at 260 mμ with an $A_{260}:A_{280}$ ratio similar to that of uridylic acid. The amount of this substance present is of the same order as the amount of pseudo-uridylic acid present on rat liver RNA (Dunn, 1959).

Five base ratio determinations were carried out, and the means of the values obtained are presented in Table 8, together with values for other yeasts given by Magasanik (1955). The small unindentified peak has been ignored.

TABLE 8

	A	G	C	U	Pu/Py
S.pombe (mean of 5 values)	10	9.5	6.5	12.0	1.06
Baker's yeast (mean of 5 values)	10	11.5	7.7	9.7	1.24
Brewer's yeast	10	10.4	9.2	9.6	1.08

The base ratios of various RNA's which have been studied depend to some extent on the method of preparation, and this should always be borne in mind when comparing one set of results with another. As far as the method of hydrolysis used here is concerned, although mild alkaline hydrolysis is used as a method for the quantitative

conversion of RNA to its nucleotides, it seems that some deamination of cytidylic acid is likely (Loring, 1955).

II. THE BIOCHEMICAL ESTIMATION OF ACID-SOLUBLE NUCLEOTIDES AND RNA IN CELL FRACTIONS

This was carried out by a modified Ogur and Rosen (1950) technique. Butanol was used for lipid extraction of the samples of homogenate or cellular sub-fractions. 1-2 mls. were added to each aliquot in a numbered centrifuge tube and the contents were thoroughly mixed and allowed to stand for 15 mins. at room temperature. The alcohol and aqueous phases were then separated by centrifugation at 1500g for 3 mins., and the top layer, which consisted of butanol saturated with the aqueous phase and containing dissolved lipid, was discarded. This process was repeated twice more. The contents of each tube were then frozen in a dry-ice and alcohol mixture (temperature -70°C.) and dried overnight in a vacuum desiccator over concentrated H_2SO_4 and KOH.

Extraction of acid-soluble nucleotides.

5 mls. of cold 2% PCA were added to each tube and the contents were thoroughly mixed and allowed to stand at 4°C for 7 mins. The tubes were then centrifuged for 3 mins. at 1500g, and the supernatent PCA was removed to a numbered McCartney bottle. This procedure was repeated, the supernatents from each tube being pooled, and the volumes of the extracts were measured. The absorption of the contents of each bottle was measured at 240, 260, 280 and 300 mμ on a Unicam spectrophotometer, to ensure that the absorption at 260 mμ was specific for nucleotides. The product of the value of the absorption at 260 mμ and the volume of the extract gave a measure of the amount of acid-soluble nucleotides present which is comparable

to similar values obtained from other samples.

Extraction of RNA

10 mls. cold 10% PCA were added to the sediment in each tube after the 2% PCA extraction. The tubes were left at 4°C for 42 hours, after which the cellular material was spun down and the extract removed to numbered McCartney bottles. The sediment was washed once with 1 ml. cold 10% PCA and the washing was pooled with the previous supernatent. Absorption measurements and the calculation of relative RNA amounts were carried out at for the acid-soluble nucleotide extracts.

A C K N O W L E D G E M E N T S

This work was carried out under the supervision of Dr P.M.B. Walker, and I should like to thank him particularly for allowing me to include the section on the organisation of cytoplasmic RNA in S. pombe, the experiments concerned being executed jointly with him as part of his research programme.

The cytophotometric measurements were made at, or with apparatus belonging to, the Medical Research Council Biophysics Research Unit, at King's College, London, and the Institute of Physiology of Glasgow University, and I am indebted to Professors Randall, F.R.S. and Garry and to members of their staffs for their generous co-operation.

Thanks are also due to many members of this department for their advice and assistance, particularly to Drs. L.J. Hale, P.D. Mitchell and J. Moyle, and to Mr. D. Cremer, who is responsible for the preparation of the prints of the diagrams and photographs.

I should also like to thank the Medical Research Council for financial support in the form of a Research Assistantship.

REFERENCES

AGAR, H.D. and H.C. DOUGLAS (1957). J. Bacteriol. 73, 365. Studies on the Cytological Structure of Yeasts: Electron Microscopy of Thin Sections.

ALLFREY, V.C. and A. MIRSKY (1957). Proc. Nat. Acad. Sci. U.S. Wash. 43, 821. Some Aspects on RNA Synthesis in Isolated Cell Nucleic.

ANDERSON, N.G. (1956). Explt. Cell Res. 11, 186. Studies on Isolated Cell Components, IX. The Soluble Phase.

ARONSON, A.L. and S. SPEIGELMAN (1958). Biochim. et Biophys. Acta. 29, 214. On the use of Chloramphenicol-inhibited systems for Investigating RNA and Protein Synthesis.

ASHIKAWA, J.K. (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis. (1st Symp. Biophys. (Soc.) p. 76. Pergamon Press. Ultracentrifugal Studies of Microsomes from Starving, Non-proliferating and Proliferating Yeast.

ATKIN, N.B. and B.M. RICHARDS (1956). Brit. J. of Cancer 10 769. Deoxyribonucleic acid in Human Tumours as Measured by Microspectrophotometry of Feulgen Stain: A Comparison of Tumours Arising at Different Sites.

ATKIN, N.B., B.M. RICHARDS and A. J. ROSS (1959). Brit. J. of Cancer, 13, 773. The DNA Constant of Carcinoma of the Uterus : An Assessment of its Possible Significance in Relation to Histo-Pathology and Clinical Course, based on data from 165 Cases.

ATTARDI, G. (1953). Experientia 9, 422. An Ultra-violet Microspectrophotmetric Study of the Purkinje Cells of the Adult Albino Rat.

ATTARDI, G. (1957). Explt. Cell Res. Suppl. 4, 25. Quantitative Behaviour of Cytoplasmic RNA in Rat Purkinje Cells Following Prolonged Physiological Stimulation.

BARER, R. (1956), in OSTER, G. and A. W. POLLISTER (eds.) Physical Techniques in Biological Research III. Cells and Tissues, p. 30. Academic Press, Inc. N.Y. Phase Contrast and Interference Microscopy in Cytology.

BARER, R. (1959) in MELLORS, R.C. (ed.) Analythical Cytology, 2nd edition. Mcgraw-Hill. Phase contrast and Interference Microscopy.

BATHER, R. (1958). Brit. J. of Cancer 12, 256. The Relationship between Infectivity and RNA Content of Partially Purified Rous Sarcoma Preparations.

BEAVAN, G.H., E.R. HOLIDAY and E.A. JOHNSON (1955) in CHARGAFF, E. and J.N. DAVIDSON (eds.) The Nucleic Acids I, p. 493. Academic Press, Inc., N.Y. Optical Properties of Nucleic Acids and their Components.

BELL, L.G.E. (1958). J. Hist. and Cytochem. 6, 435. The Site of a Diffusible Nucleotide Revealed by a Comparison of Chemically fixed with Frozen and Dried Tissue.

BELL, L.G.E. (1959). Exptl. Cell Res. 16, 615. The Combination of a Portion of the Cytoplasmic RNA Compounds with Mercury.

BERG, P. and E.J. OFENGAND (1958). Proc. Nat. Acad. Sci. U.S. Wash. 44, 78. An Enzymatic Mechanism for Linking Amino-Acids to R.N.A.

BIRBECK, MSC. and E. REID (1956). Biochem. et Biochys. Acta 20, 419. A New Medium for the Isolation of Liver Mitochondria.

BOLTON, E.T., B.H. HOYER and D.B. RITTER. (1958) in ROBERTS, R.B. (ed). Microsomal Particles and Protein Synthesis. (1st. Symp. Biophys. Soc.), p. 19. Pergamon Press. Stability of Ribonucleoprotein Particles of Escherichia coli.

BOWEN, T.J., S. DAGLEY & J.SYKES (1959) Biochem. J. 71 : 2, 14P. A. Ribonucleoprotein Component of Escherichia coli.

BOWEN, T.J., S. DAGLEY & J.SYKES (1959) Biochem. J. 72, 419. A Ribonucleoprotein Component of Escherichia coli.

BRACHET, J. (1942). Arch. de Biol. 53, 207. La Localisation des acides Pentosenucléiques dans les Tissus Animaux at les Oeufs d' Amphibiens en Voie de Developpement.

BRACHET, J. (1957). Biochemical Cytology, Academic Press, Inc. N.Y.

BRACHET, J. (1959). Biochim. et Biophys. Acta 35, 580. New Observations on the Mode of Action of Ribonuclease on Living Root Tips.

BRACHET, J. and R. JEENER (1944) Enzymologia 11, 196. Recherches sur des Particles Cytoplasmiques de Dimensions Macromoléculaires Riches en Acide Pentosenucléique.

BRATTGARD, S. and H. HYDÉN, (1954) in BOURNE, G. and J.F. DANIELLI (eds.) Int. Rev. of Cytol. 3. The Composition of the Nerve Cell Studied with New Methods.

BRATTGARD, S., J-E. EDSTROM and H. HYDÉN (1957). J. of Neurochem. 1, 316. The Chemical Changes in regenerating Neurones.

BRATTGARD, S., J-E. EDSTROM and H. HYDÉN (1958). Exptl. Cell Res. Suppl. 5, 185. The Productive Capacity of the Neurone in Retrograde Reaction.

BRITTEN,R.J., K.G., LARK and F.NORCROSS (1960). Biochim.et Biophys. Acta 39, 150. Sedimentation Characteristics of Bacterial Ribonucleoprotein Obtained at Different Periods of the Cell-division Cycle.

CASPERSSON,T. (1936). Skand.Arch. Physiol. 73, Suppl. 8. Uber den Chemischen Aufbau der Struktures des Zellkernes.

CASPERSSON, T. (1947). Symp. Soc. Exptl. Biol. 1, 127. The Relation between Nucleic Acid and Protein Synthesis.

CASPERSSON, T. (1950). Cell Growth and Cell Function. W.W. Norton and Co.,Inc.

CASPERSSON, T. (1955). Experientia 11, 45. Quantitative Cytochemical Methods for the Study of Cell Metabolism.

CASPERSSON, T. (1956). Cold Spring Harbor Symp. on Quant. Biol. 21, 1. Quantitative Cytochemical Determinations on Endonuclear Structures.

CASPERSSON, T. (1957). Exptl. Cell Res. Suppl. 4, 3. A Project for the Development of Quantitative Methods for the Endocellular Range.

CASPERSSON, T., G. LOMAKKA and G. SVENSON, (1957). Exptl. Cell. Res. Suppl. 4. A Co-ordinated Set of Instruments for Optical Quantitative High Resolution Cytochemistry.

CASSELL, W.A. (1950-. J.Bacteriol. 59, 185. The use of Perchloric Acid in Bacterial Cytology.

CHAO, F-C. (1957). Arch. Biochem. and Biophys. 70, 426. Dissociation of the Macromolecular Ribonucleoprotein of Yeast.

CHAO, F-C and H.K.SCHACHMAN (1956). Arch. Biochem, and Biophys. 61, 220. The Isolation and Characterisation of a Macromolecular Ribonucleoprotein from Yeast.

CHAPMAN, G.B. (1959). J. Biophys. and Biochem.Cytol. 6, 221. Electron Microscope Observations on the Behaviour of the Bacterial Cytoplasmic Membrane during Division.

CHAPMAN, G.B. and J. HILLIER (1953). J. Bacteriol. 66, 362. Electron Microscopy of Ultra-thin Sections of Bacteria. I. Cellular Division in Bacillus cereus.

CLAUDE, A (1943). Science 97, 451. The Constitution of Protoplasm.

COMMONER, B. (1949). Science 110, 31. On the Interpretation of the Absorption of Ultra-violet light by Nucleic Acid.

DAGLEY, S. and J. SYKES (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st. Symp. Biophys. Soc.) p. 62. Pergamon Press. The Influence of Conditions of Culture on Certain Soluble Marcromolecular Components of Escherichia coli

DAGLEY, S. and J. SYKES (1960). Biochem. J. 74 11P. Bacterial Ribonucleoprotein Synthesis in the Presence of Chloramphenicol.

DANIELLI, J.F. (1953). Cytochemistry. Chapman Hall, Ltd., London.

DAVIDSON, J.N. (1953). The Biochemistry of the Nucleic Acids. 1st. edition. Methuen and Co., Ltd., London.

DAVIES, H.G. (1950). Gen. Disc. of the Faraday Soc. Spectroscopy and Molecular structure and Optical Methods of Investigating Cell Structure, p. 442. Ultra-violet Microspectography of Living Tissue Culture Cells. I. Radiation Measurements.

DAVIES, H.G. (1954). Quart. J. Micr. Sci. 95, 433. The Action of Fixatives on the Ultra-violet Absorbing Components of Chick Fibroblasts.

DAVIES, H.G. in DANIELLI, J.F. (ed.) General Cytochemical Methods 1, 57. Academic Press. The determination of Mass and Concentration by Microscope Interferometry.

DAVIES, H.G. E.M.DEELEY and E.F. DENBY (1957). Exptl. Cell. Res. Suppl. 4, 136. Attempts at Measurement of the Lipid, Nucleic Acid and Protein Content of Cell Nuclei by Microscope Interferometry.

DAVIES, H.G. and A. ENGSTROM (1954). Exptl. Cell Res. 7, 243. Interferometric and X-ray absorption Studies of Bone Tissue.

DAVIES, H.G., A. ENGSTROM and B. LINDSTROM (1953). Nature, 172, 1041. A Comparison between the X-ray Absorption and Optical Interference Methods for the Mass Determination of Biological Structures.

DAVIES, H.G., M.H.F. WILKINS, J. CHAYENS and L.F. la COUR (1954). Quart. J. Micr. Sci. 95, 271. The Use of the Interference Microscope to Determine Dry Mass in Living Cells and as a Quantitative Cytochemical Method.

DAVIES, H.G. and P.M.B. WALKER (1953). Progress in Biophysics 3, 195. Microspectrophotometry of Living and Fixed Cells.

DAVIES, H.G., M.H.F. WILKINS and R.H.G.B. BODDY (1954). Exptl. Cell Res. 6, 550. Cell Crushing: a Technique for Greatly Reducing Errors in Microspectrometry.

van der DECKEN, A. and T. HUTLIN (1959). Exptl. Cell Res. 17, 188. The Effect of Ribonuclease on the Amino-acid Dependent Pyrophosphate-ATP exchange by the 'pH 5' fraction of Rat Liver.

DEELEY, E.M. (1955). J. Sci. Instr. 32, 263. An Integrating Microdensitometer for Biological Cells.

DEELEY, E.M., B.M. RICHARDS, P.M.B. WALKER and H.G. DAVIES, (1954). Exptl. Cell Res. 6, 569. Measurements of Feulgen Stain during the Cell Cycle with a New Photolectric Scanning Device.

DINTZIS, H.M., H. BORSOOK and J. VINOGRAD (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis. (1st. Symp. Biophys. Soc.) p. 95 Pergamon Press. Microsomal Structure and Haemoglobin Synthesis in the Rabbit Riticulocyte.

DOTY, P., H. BOEDGKER, J.R. FRESCO, R. HASELKORN, & M. LITT. (1959) Proc. Nat. Acad. Sci. U.S.A.Wash. 45, 482. Secoondary Structure in Ribonucleic Acid.

DUNN, D.B. (1959) Biochim. et Biohys. Acta. 34, 296. Additional Components in the RNA of Rat-Liver Fractions.

EDSTROM, J-E (1953). Biochim. et. Biophys. Acta. 12, 361. RNA Mass and Concentration in Individual Nerve Cells.

EDSTROM, J-E (1956). Biochim. et. Biophys. Acta. 22, 378. Separation and Determination of Purines and Pyrimidines in Picogram Amounts.

EDSTROM, J-E (1958a) of Neurochem. 3, 100. Quantitative Determination of RNA in the uugm. range.

EDSTROM, J-E (1958b). Microchem, J. 2, 71. Determination of Organic Compounds below the Microgram Range. RNA and its Components.

EDSTROM, J-E.and D. EICHNER (1958a). Nature 181, 619. Relation between Nucleolar Volume and Cell Body Content of RNA in Supra-optic Neurones.

EDSTROM, J-E. and D. EICHNER (1958b). Z. fur Zellforchung und Mikroskopische Anatomie 48, 187. Quantitative Ribonukleinsaure-untersuchungen an der gangliezellen des nucleus supraopticus der albino-ratte unter experimentellen bedingungen (koch-salz-belestung).

EDSTROM, J-E. and A. PIGON (1958). J. of Neurochem. 3, 95. Relation Between Surface, RNA Content and Nuclear Volume in Encapsulated Spinal Ganglion Cells.

ELSON, D. (1959) Biochim. et Biophys. Acta 36, 362. Preparation and Properties of a Ribonucleoprotein Isolated from Escherichia coli.

ELSON, D. and E. CHARGAFF (1955). Biochim. et Biophys. Acta 17, 367. Evidence of Common Regularities in the Composition of Pentose Nucleic Acids.

ELSON, D. and M. TAL (1959). Biochim. et Biophys. Acta 36, 281. Biochemical Differences in Ribonucleoproteins.

ENGSTROM, A. (1956) in OSTER, G. and A.W. POLLISTER (eds.) Physical Techniques in Biological Research, III Cells and Tissues, p. 489. Academic Press, Inc. N.Y. Historadiography.

ENGSTROM, A. (1959) in MELLORS, R.C. (ed.) Analytical Cytology, 2nd edition. McGraw-Hill X-ray Microscopy.

ENGSTROM, A. and B. LINDSTROM (1958) in DANIELLI, J.F. (ed.) General Cytochemical Methods I, 1. Academic Press, Inc. N.Y. The Weighing of Cell Structures by Ultra-soft X-rays.

FAED, M.J.W. (1959) Ph.D. Thesis, University of Edinburgh.

FIRKET, H. (1958) Arch. de. Biol. 64, 1. Recherches sur la synthese des ADN et la preparation a la mitose dans les cellules cultivees 'in vitro'. (Etude cytophotometrique et autoradiographique).

FLAX, M.H. and M.H. HIMES. (1952). Physiol. Zool. 25, 297. Microspectrophotometric Analysis of Metachromatic Staining of Nucleic Acids.

de FONBRUNE, P. (1949). Monographies D'Institut Pasteur, Masson et Cie, Paris. Technique De Micromanipulation.

GIERER, A. and K.W. MUNDRY (1958). Nature 182, 1457. Production of Mutants of T.M.V. by Chemical Alteration of its RNA in vitro.

GILCHREIST, W.C. and R.M. BOCK (1958). in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st Symp. Biophys. Soc.) p. 1. Pergamon Press. Isolation and Characterisation of Bacterial Nucleoprotein Particles.

GORDON, M.W. and J.I. NURNBERGER (1955). Exptl. Cell Res. 8, 279. Acute Cold-induced Changes in Cellular Protein and Nucleic Acids in Rat Liver and Brain.

GREENSTEIN, J.P., C.E. CARTER and H.W. CHALKLEY (1947). Cold Spring Harbor Symp. on Quant. Biol. 12, 64. Enzymatic Degradation of Ribonenucleic and Desoxyribosenucleic Acids with an Addendum on the Effects of Nucleates on the Heat Stability of Protein.

HAKIM, A.A. (1957). Arch. Biochem. and Biophys. 70, 591. Enzymic Heterogeneity of Crystalline Ribonuclease.

HALE, L.J. (1958). Biological Laboratory Data. p. 75. Methuen and Co.

HALL, B.D. and P. DOTY (1959). J. Molec. Biol. 1, 111. The Preparation and Physical Chemical Properties of RNA from Microsomal Particles.

HALL, C.E. and H.S. SLAYTER (1959). J. Molec. Biol. 1, 329. Electron Microscopy of RNP Particles from Escherichia coli.

HANSEN, I.A. and P.M. NOSSAL (1959). Biochem. et Biophys. Acta 16, 502. Morphological and Biochemical Effects of Freezing on Yeast Cells.

HANZON, V., L.H. HERMODSSON and G. TOSCHI (1959). J.,of Ultrastruct. Res. 3, 216. Ultra-structural Organisation of Cytoplasmic Nucleoprotein in the Exocrine Pancreatic Cells.

HARNDEN, D.G. (1957). Ph.D. Thesis, University of Edinburgh.

HARVEY, E.B. (1940). Biol. Bull. 79, 166. A Comparison of the Development of Nucleate and Non-nucleate Eggs of Arbacia punculata.

HASHIMOTO, T., S.F. CONTI and H.B. NAYLOR (1959). J. Bacteriol. 77, 344. Fine Structure of Micro-organisms 4. Observations on Budding Saccharomyces cerevisiae by Light and Electron Microscopy.

HESS, E.L.and S.E. LAGG (1958). J. Biophys. and Biochem. Cytol. 4, 717. Composition of Calf Thymus. A Comparison of Differential Centrifugation and Chemical Fractionation Procedures.

HEYES, J.K. (1960). Proc. Roy. Soc. (B). 152, 218. Nucleic Acid Changes during Cell Expansion in the Root.

HIGUCHI, M. and T. UEMURA (1959). Nature 184, 1381. Release of Nucleotides from Yeast Cells.

HOAGLAND, M.B. (1959). Scientific American 201, 55. Nucleic Acids and Proteins.

HOAGLAND, M.B. and P.C. ZAMECNIK (1957). Fed. Proc. 16, 197. Intermediate Reactions in Protein Synthesis.

HOAGLAND, M.B., P.C. ZAMECNIK and M.L. STEVENSON (1957). Biochim et Biophys. Acta 24, 215. Intermediate Reactions in Protein Synthesis.

HOGEBOOM, G.H., and W.C.SCHNEIDER (1955) in CHARGAFF, E. and J.N. DAVIDSON (eds.) The Nucleic Acids II, Academic Press, Inc. N.Y. p. 199. The Cytoplasm.

HOLTER, H. (1954). Proc. Roy. S c. (B) 142, 140. Distribution of Some Enzymes in the Cytoplasm of Some Amoebae.

HOTTA, Y. and S. OSAWA (1958). Biochim. et Biophys. Acta. 28, 642. Further Studies on Nuclear and Cytoplasmic Nucleic Acids.

HOWATSON, A.F. and A.W. HAM (1955). Canc. Res. 15, 62. Electron Microscope Study of Section of Two Rat Liver Tumours.

HUGHES, D.E. (1951). Brit. J. of Exptl. Path. 32, 97. A Press for Disrupting Bacteria and Other Micro-organisms.

HURLBERT, R.B., H. SCHMIDT, A.F. BRUMM and V.R. POTTER (1954). J. Biol. Chem. 209, 23. Nucleotide Metabolism II Chromatographic Separation of Acid-soluble Nucleotides.

HUXLEY, H.E. and G. ZUBAY (1960). J. Molec. Biol. 2, 10. Electron Microscope Observations on the Structure of Microsomal Particles from Escherichia coli.

HYDÉN, H. (1959). in the IVth Inter. Congress of Biochem. (Vienna, 1958) III Biochemistry of the Nervous System. p. 64. Pergamon Press, London.

IMMERS, J. (1960). Exptl. Cell Res. 19, 499. Studies on Cytoplasmic Components of Sea Urchin Eggs Stratified by Centrifugation.

IWAMURA, T. and J. MYERS (1959). Arch. Biochem, and Biophys. 84, 267. Changes in the Content and Distribution of the Nucleic Acid Bases in Chlorella during the Life Cycle.

KIMBALL, R.F., T.O. CASPERSSON, G. SVENSSON and L. CARSON (1959) Exptl. Cell Res. 17, 160. Quantitative Cytochemical Studies on Paramecium aurelia.

KING, R.J. (1959). Quart. J. Micr. Sci. 100, 25. A Quantitative Study of the Cytoplasm of Ascites Tumour Cells in Mice.

KING, R.J. and E.M. ROE (1953). J. Roy. Micr. Soc. 73, 82. Optical Conditions for Quantitative Ultra-violet Spectroscopy.

KIT, S. (1960). Arch. Biochem. and Biophys. 88, 1. Base Composition of the Ribonucleic Acids of Normal Tissues, Tumours and Tissue Subfractions.

KLEINSCHMIDT, W.J. (1959). Canc. Res. 19, 966. The Composition of RNA and DNA of Normal and Neoplastic Tissue.

KORNE, D. and D.H. NORTHCOTE, (1960). Biochem. J. 75, 12. Physical and Chemical Properties of Polysaccharides and Glycoproteins of the Yeast Cell Wall.

LACKS, S. and F. GROS (1959). J. Molec. Biol. 1, 301. A Metabolic study of the RNA-Amino-Acid Complexes in Escherichia coli.

de LAMIRANDE, G., C. ALLARD and A. CANTERO (1955). Canc. Res. 15, 329. RNA Composition of Rat Liver induced by 4-dimethylaminoazobenzene.

de LAMIRANDE, G., C. ALLARD and A. CANTERO (1958). Biochim. et Biophys. Acta 27, 395. Intracellular RNA Composition in Regenerating Liver Cells.

LESLIE, I. (1955). in CHARGAFF, E., and J.N. DAVIDSON (eds.) The Nucleic Acids II, p. 1. Academic Press, N.Y. The Nucleic Acid Content of Cells and Tissues.

LEUCHTENBERGER, C.R., LEUCHTENBERGER, C. VENDRELY and R. VENDRELY (1952) Exptl. Cell Res. 3, 240. Quantitative Estimation of DNA in Isolated Individual Animal Nuclei by the Caspersson Ultraviolet Method and Its Comparison with Biochemical Estimation.

LEUCHTENBERGER, C., G. KLEIN and E. KLEIN (1952). Canc. Res. 12, 480. The Estimation of Nucleic Acids in Individual Isolated Nucleic of Ascites Tumours by Ultra-violet Microspectrophotometry and its Comparison with Chemical Analysis.

LEUCHTENBERGER, C., R. VENDRELY and C. VENDRELY (1951), Proc. Nat. Acad. Sci. U.S. Wash. 37, 33. A Comparison of the Content of DNA in Isolated Animal Nuclei by Cytochemical and Chemical Methods.

LEVENBOOK, L., E.L. TRAVAGLINI and J. SCHULTZ (1958). Exptl. Cell Res. 15, 43. Nucleic Acids and Their Components as Affected by the Y Chromosome of Drosophila melanogaster 1. Constitution and Amounts of the Ribonucleic Acids in the Unfertilised Egg.

LIPMANN, F., W.C. HULMANN, G. HARTMANN, H.G. BOMAN, and G.ACS (1959) J. Cell. and Comp. Physiol. 54, Suppl. 1. Amino-acid Activation and Protein Synthesis.

LITTLEFIELD, J.M. and D.B. DUNN (1958a) Biochem. J. 68, 1, 8P. Occurrence and Distribution of Thymine and Methylated Adenine Bases in Ribonucleic Acids.

LITTLEFIELD, J.M. and D.B. DUNN (1958b). Nature, 181, 254./Natural Occurrence of Thymine and Three Methylated Bases in Several Ribonucleic Acids.

LOFTFIELD, R.B. (1957). Progress in Biophysics and Biophysical Chemistry 8, 348. The Biosynthesis of Protein.

LORING, H.S. (1955), in CHARGAFF, E. and J. N. DAVIDSON (eds.) The Nucleic Acids, I, p. 191. Academic Press, Inc., N.Y. Hydrolysis of Nucleic Acids and Procedures for the Direct Estimation of Purine and Pyrimidine Fractions by Absorption Spectrophotometry.

LOW, E.M. (1958). Nature 182, 1096. Composition of the Nucleic Acids of Some Algae.

LOWE, C.U. and A.L. LEHNINGER (1955). J. Biophys. and Biochem. Cytol. 1, 89. Oxidation and Phosphorylation in Liver Mitochondria lacking "Polymerized" RNA.

MAGASANIK, B. (1955), in CHARGAFF, E. and J.N. DAVIDSON (eds.) The Nucleic Acids I, p. 373. Academic Press, Inc., N.Y. Isolation and Composition of the Pentose Nucleic Acids and of the Corresponding Nucleoproteins.

MAGER, J. and F. LIPMANN (1958). Proc. Nat. Acad. Sci. U.S. Wash. 44, 305. Amino-acid Incorporation and the Reversion of its Initial Phases with Cell-free Tetrahymena Preparations.

MARKHAM, R., and J.D. SMITH (1952). Biochem. J. 52, 565. The Structure of RNA. 3. The End Groups, the General Structure and the Nature of the 'Core'.

MARUYAMA, Y. and K.G. LARK (1959). Exptl. Cell Res. 18, 389. Periodic Synthesis of RNA in Synchronized Cultures of Alcaligenes faecalis.

MARUYAMA, Y. and T. YAMAGITA (1956). J. Bacteriol. 71, 542. Physical Methods for Obtaining Synchronous Cultures of Escherichia coli.

McCARTHY, B.J. (1960). Biochim. et Biophys. Acta 39, 563. Variation in Bacterial Ribosomes.

McMASTER-KAYE, R. and J.H. TAYLOR (1958). J. Biophys. and Biochem. Cytol. 4, 5. Evidence for Two Metabolically Distinct Types of RNA in Chromatin and Nucleoli.

MENDELSOHN, M.L. and B.M. RICHARDS (1958). J. Biophys. and Biochem. Cytol, 4, 707. A Comparison of Scanning and Two-Wavelength Microspectrophotometry.

MICHAELIS, L. (1947). Cold Spring Harbor Symp. on Quant. Biol. 12, 131. The Nature of the Interaction of Nucleic Acids and Nuclei with Basic Dye-stuffs.

MINAGAWA, T (1957). Exptl. Cell Res. 13, 224. Adaptation of Yeast to Copper XIV. Qualitative Change of RNA in Yeast Cultured in Copper Medium.

MITCHELL, P. and J. MOYLE (1951). J. Gen. Microbiol. 5, 981. The Glyco-protein Complex Envelope of Micrococcus pyogenes.

MITCHISON, J.M. (1957). Exptl. Cell Res. 13, 244. Growth of Single Cells 1. Schizosaccharomyces pombe.

MITCHISON, J.M. and P.M.B. WALKER (1959). Exptl. Cell Res. 16, 49. RNA Synthesis during the Cell Life Cycle of a Fission Yeast Schizosaccharomyces pombe.

MOBERGER, G. (1954). Acta Radiol. Scand. Suppl. 112. Malignant Transformation of Squamous Epithelium.

MOSES, M.J. (1952). Exptl. Cell Res. Suppl. 2. Optical Techniques in Nuclear Chemistry.

NEWTON, A.A. and P. WILDY (1959). Exptl. Cell Res. 16, 1024. Parasychronous Division of HeLa Cells.

NOVIKOFF, A.B. (1956). Fed. Proc. 15, 322. Mitochrondria Isolated from Polyvinylpyrollidone-sucrose Homogenates of Rat Liver.

NURNBERGER, J.I. (1955) in MELLORS, R.C. (ed.) Analytical Cytology, 1st. edition. McGraw-Hill. Ultra-violet Microscopy and Microspectroscopy

NURNBERGER, J.I., A. ENGSTROM and B. LINDSTROM (1952). J. Cell. and Comp. Physiol. 39, 215. A Study of the Ventral Horn Cells of the Adult Cat by Two Independent Cytochemical Microabsorption Techniques.

OGUR, M. and G. ROSEN (1950). Arch. Biochem and Biophys. 25, 262. The Nucleic Acids of Plant Tissues. I. The Extraction and Estimation of DNA and PNA.

OGATA, K. and H. NOHARA (1957). Biochim. et Biophys Acta 25, 659. A Possible Role of the RNA of the pH 5 Enzyme in Amino-Acid Activation.

ORNSTEIN, L. (1952). Lab. Invest. 1, 250. The Distributional Error in Microspectrophotometry.

OSAWA, S., K. TAKATA and Y. HOTTA (1958). Biochim. et Biophys. Acta 28, 271. Nuclear and Cytoplasmic Ribonucleic Acids of Calf Thymus.

OTAKA, E., and S. OSAWA (1960). Nature 185, 921. Preparation and Some Properties of a Soluble RNA from Yeast.

PALADE, G.E. (1958). in ROBERTS,R.B. (ed.) Microsomal Particles and Protein Synthesis (1st Symp. Biophys. Soc.), p. 36. Pergamon Press. Microsomes and Ribonucleoprotein Particles.

PALADE, G.E., and P. SIEKEVITZ (1956). J. Biophys. and Biochem. Cytol. 2, 171. Liver Microsomes. An Integrated Morphological and Biochemical Study.

PATAU, K. (1952). Chromosoma 5, 341. Absorption Microphotometry of Irregular-shaped Objects.

PETERMANN, M.L. and M.G. HAMILTON (1952). Canc. Res. 12, 373. An Ultracentrifugal Analysis of the Macromolecular Particles of Normal and Leukaemic Mouse Spleen.

PETERMANN, M.L. and M.G. HAMILTON (1958a) Abstract 4. Inter. Congress of Biochem., Vienna, p. 34.

PETERMANN, M.L. and M.G. HAMILTON (1958b). J. Biophys. and Biochem. Cytol, 4, 771. The Influence of Age, Sex, Pregnancy, Starvation and Other Factors on the Cytoplasmic Ribonucleoproteins of Rat Liver.

PETERMANN, M.L. M.G. HAMILTON, M.E. BALIS, K. SAMMTH, and P. PECORA, (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st. Symp. Biophys Soc.), p. 70. Pergamon Press. Physiochemical and Metabolic Studies on Rat Liver Ribonucleoprotein.

POLLISTER, A.W. and L. ORNSTEIN (1959) in MELLORS, R.C. (ed.) Analytical Cytology, 2nd edition, McGraw-Hill. The Photometric Chemical Analysis of Cells.

PRESCOTT, D.M. (1960). Exptl. Cell Res. 19, 228. Relation Between Cell Growth and Cell Division. IV. The Synthesis of DNA, RNA and Protein from Division to Division in Tetrahymena.

QUATTRONE, P.D. (1959). Exptl. Cell Res. 16, 506. Comparison of the Nucleic Acid Content in Various Tissues of Mice.

RASCH, E., H. SWIFT and R.M. KLEIN (1959). J. Biophys. and Biochem. Cytol. 6, 11. Nucleoprotein Changes in Plant Tumour Growth.

REID, E. and B.M. STEVENS, (1958). Nature 182, 441. Ribonucleic Acids in Liver Cytoplasm.

RICHARDS, B.M., P.M.B. WALKER and E.M. DEELEY (1956). Ann. N.Y. Acad. Sci. 63, Art. 5, 831. Changes in Nuclear DNA in Normal and Ascites Tumour Cells.

RICHARDS, B.M. and N.B. ATKIN (1959). Brit. J. Canc. 13, 788. DNA Content of Human Tumours. Changes in Uterine Tumours during Radiotherapy and Their Response to Treatment.

RIS, H. and A.E. MIRSKY (1950). J. Gen. Physiol. 33, 125. Quantitative Cytochemical Determination of DNA with the Feulgen Nucleal Reaction.

ROBERTS, R.B. (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st Symp. Biophys. Soc.) Pergamon Press.

ROBERTS, R.B., R.J. BRITTEN and E.T. BOLTON (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st. Symp. Biophys. Soc.) p. 84, Pergamon Press. Fractionation of Escherichia coli for Kinetic Studies.

RUDKIN, G.T. and S.L. CORLETTE (1957). Proc. Nat. Acad. Sci. U.S. Wash. 43. 964. Disproportionate Synthesis of DNA in a Polytene Chromosome Region.

RUSTAD, R.C. (1959). Exptl. Cell Res. 16, 575. An Interference and Cytochemical Method Analysis of Local Mass Changes in the Mitotic Apparatus during Mitosis.

SCHERBAUM, O. (1957a).Exptl. Cell Res. 13, 11. Studies on the Mechanism of Synchronous Cell Division in Tetrahymena pyriformis.

SCHERBAUM, O. (1957b).Exptl. Cell Res. 13, 24. The Content and Composition of Nucleic Acids in Normal and Synchronously Dividing Mass Cultures of Tetrahymena pyriformis.

SCHMIDT, G. (1955). in CHARGAFF, E. and J.N. DAVIDSON (eds.) The Nucleic Acids I, p. 555. Academic Press, Inc., N.Y. Nucleases and Enzymes Attacking Nucleic Acid Components.

SCHWEET, R.S., F.C. BOVARD, E. ALLEN and E. GLASSMAN (1958). Proc. Nat. Acad. Sci. U.S. Wash. 44, 173 The Incorporation of Amino-acids into RNA.

SHIGEURA, H.T. and E. CHARGAFF (1958). Biochim. et Biophys. Acta 30, 434. Fractionation of the 'Soluble' RNA of Rat Liver Cytoplasm.

SHIGEURA, H.T. and E. CHARGAFF (1960). Biochim et Biophys Acta 37, 347. Action of Ribonuclease on Microsomal Ribonucleoprotein.

SIEKEVITZ, P. (1959). Exptl. Cell Res. Suppl. 7. The Cytological Basis of Protein Synthesis.

SIMPSON, M.V., J.R. McLEAN, G.L. COHEN and I.K. BRANDT (1957) Fed. Proc. 16, 249. In Vitro Incorporation of Leucine-I-C^{14} into the Protein of Liver Mitochondria.

SIRLIN, J.L. (1960). Nature 186, 275. The Nucleolus Problem.

SPAHR, P.F. and A. TISSIERES (1959). J. Molec. Biol. 1, 237. Nucleotide Composition of RNP Particles from Escherichia coli.

STENRAM, U. (1958a) Acta Path. and Microbiol. Scand. 44, 239. Volume and RNA Concentration of Nucleoli in Liver and Hepatoma Cells of Rats on High-and-Non-protein Diets.

STENRAM, U. (1958b). Exptl. Cell Res. 15, 174. Interferometric Determination of the RNA Concentration in Liver Nucleoli of Protein-fed and Protein-deprived Rats.

STICH, H.F. and J. McINTYRE (1958). Exptl. Cell Res. 14, 635. X-ray Absorption Studies on the Nuclear Protein and RNA Content during the Development of the Mitotic Apparatus.

SWIFT, H. (1955) in CHARGAFF, E. and J.N. DAVIDSON (eds.) The Nucleic Acids, II, p. 51. Academic Press, Inc. N.Y. Cytochemical Techniques for the Nucleic Acids.

SWIFT, H. (1958) in McELROY, W.D. and B.GLASS (eds). The Chemical Basis of Development. John Hopkins Press. Cytoplasmic Particulates and Basiphilia.

SWIFT, H. and E. RASCH, (1956) in OSTER, G. and A.W. POLLISTER (eds.) Physical Techniques in Biological Research III. Cells and Tissues, p. 354. Academic Press, Inc., N.Y. Microspectrophotometry in Visible Light.

SWIFT, H., L. REBHUN, E. RASCH and J. WOODARD (1956), in RUDNICK, D. (ed.) Cellular Mechanisms in Differentiation and Growth (14th. Growth Symp.) The Cytology of Nuclear RNA.

SYLVÉN, B. (1952) in Freezing and Drying, p. 169. The Institute of Biology, London.

SYLVÉN, B. (1954) Quart. J. Micr. Sci. 95, 327. Metachromatic Dye-Substrate Interactions.

TISSIERES, A. (1959). J. Molec. Biol. 1, 365. Some Properties of the Soluble RNA from Escherichia coli.

TISSIERES, A. and J.D. WATSON (1958). Nature 182, 778. Ribonucleoprotein Particles from Escherichia coli.

TISSIERES, A., J.D. WATSON, D. SCHLESSINGER and B.R. HOLLINGSWORTH(1959) J. Molec. Biol. 1, 221. Ribonucleoprotein Particles from Escherichia coli.

TRAVAGLINI, E.C., L. LEVENBOOK and J. SCHULTZ (1958) Exptl. Cell Res. 15, 62. Nucleic Acids and Their Components as Affected by the Y Chromosome of Drosophila melanogaster II. Nucleosides and Related Compounds in the Acid-Soluble Fraction of the Unfertilised Egg.

T'SO, P.O.P. (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis. (1st. Symp. Biophys. Soc.), p. 156. Pergamon Press. Structure of Microsomal Nucleoprotein Particles from Pea Seedlings.

WADE, H.E. andD.M. MORGAN (1957) Biochem. J. 65, 321. The Nature of the Fluctuating RNA in Escherichia coli.

WAGMAN, J. and W.R. TRAWICK (1958) in ROBERTS, R.B. (ed.) Microsomal Particles and Protein Synthesis (1st. Symp. Biophys. Soc.) p. 11. Pergamon Press. The Stabilization and Physical Characteristics of Purified Bacterial Ribonucleoprotein Particles.

WALKER, P.M.B. (1954). J. Exptl. Biol. 31, 8. The Mitotic Index and Interphase Processes.

WALKER, P.M.B. (1955). Exptl. Cell Res. 8, 567. A High Speed Recording Microdensitometer Suitable for Cytological Research.

WALKER, P.M.B. (1956) in OSTER, G. and A.W. POLLISTER (eds.) Physical Techniques in Biological Research, III. Cells and Tissues, p. 402. Academic Press, Inc., N.Y. Ultra-violet Absorption Techniques.

WALKER, P.M.B. (1957). Exptl. Cell Res. Suppl. 4. The Ultra-violet Absorptivities of DNA and Other Substances in Cells.

WALKER, P.M.B. (1958) in DANIELLI, J.F. (ed.) General Cytochemical Methods 1, p. 164 Academic Press, Inc. N.Y. Ultra-violet Microspectrophotometry.

WALKER, P.M.B. and H.G. DAVIES (1950) Gen. Disc. Faraday Soc. Spectroscopy and Molecular Structure and Optical Methods of Investigating Cell Structure, p. 461. Microspectrographic Studies of Living Cells and Ultra-violet Irradiated Chick Fibroblasts.

WALKER, P.M.B. and B.M. RICHARDS (1957) Exptl. Cell Res. Suppl. 4. A Method for Investigating the Stoichiometry of Feulgen Stain.

WALKER, P.M.B: and B.M. RICHARDS (1958) in BRACHET, J. and A.E. MIRSKY (eds.) The Cell I, p. 91. Academic Press, Inc., N.Y. Quantitative Microscopical Techniques for Single Cells.

WALKER, P.M.B. and H. YATES (1952a) Soc. Exptl. Biol. Symp. 6. Ultra-violet Absorption of Living Cell Nuclei during Growth and Division.

WALKER, P.M.B. and H.YATES (1952b). Proc. Roy.Spc. (B) 140, 274. Nuclear Components of Dividing Cells.

WEBSTER, G.C. (1957a) Fed. Proc. 16, 267. Amino-acid Incorporation into the Proteins of Disrupted Ribonucleoprotein Particles.

WEBSTER, G.C (1957b). Arch. Biochem. and Biophys. 70, 622. Factors Required for Amino-acid Incorporation by Disrupted Ribonucleoprotein Particles.

WEISS, S.B. (1958). Proc. Nat. Acad. Sci. U.S. Wash. 44, 189. Amino-Acid Incorporation in Pigeon Pancreas Fractions.

WILKINS, M.H.F. (1953) J.Roy. Micr. Soc. 73: 2, 77 The Performance of Spherical Mirror Reflecting Objectives when Used for Ultra-violet Photomicrography.

WOLFE, R.G. (1956). Arch. Biochem. and Biophys. 63, 100. Macromolecular Constituents of Proliferating and Non-proliferating Yeast Extracts.

WOODARD, J.W. (1958). J. Biophys. and Biochem. Cytol. 4, 383. Intracellular Amounts of Nucleic Acids and Protein during Pollen Grain Growth in Tradescantia.

ZALOKAR, M. (1960) Exptl. Cell Res. 19, 114. Cytochemistry of Centrifuged Hyphae of Neurospora.

ZAMECNIK, P.C., M.L.STEVENSON and L.I.HECHT (1958). Proc. Nat. Acad. Sci. Wash. N.Y. 44, 73. Intermediate Reactions in Protein Synthesis.

ZITTLE, C.A. (1946). J. Biol. Chem. 163, 111. The Behaviour of Copper and Calcium in the Purification of Nucleic Acid and the Effect of These and Other Reagents on the Activity of Ribonuclease.