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ABSTRACT OF THESIS

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Title of Thesis Computer Applications to the Study of Developmental Processes.

This thesis is an attempt to show how computer models may be used to help us better understand the mechanisms of development. It is divided into two main sections. The first consists of three chapters explaining (1) the importance and role of model building in biology, and the ways in which such methodologies can be of use in developmental biology; (2) computer methods used by the author; and (3) a review of the previous literature relevant to this approach.

The second section deals mainly with model systems I have used based on the ideas expounded in the first part of the thesis. The fourth chapter comprises a statistical and computer study of the analysis of patches and clones seen in chimaeras and X-inactivation mosaics. The next two chapters deal with the development of a specific system, the Drosophila head disc, and introduce models relating to disc morphogenesis and differentiation. Data on the dosing series of the Drosophila mutant bar have been used to construct models suggesting how differentiation in both wild type and bar individuals is activated.

The last chapter discusses the usefulness of the techniques outlined in the previous two sections.

COMPUTER APPLICATION TO DEVELOPMENTAL PROCESSES

by

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Thesis presented for the
Degree of Doctor of Philosophy
of the University of Edinburgh
in the Faculty of Science.

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PART ONE

CONCEPTS AND METHODOLOGIES

SUMMARY

This thesis is an attempt to show how computer models may be used to help us better understand the mechanisms of development. It is divided into two main sections. The first consists of three chapters explaining (1) the importance and role of model building in biology, and the ways in which such methodologies can be of use in developmental biology; (2) computer methods used by the author; and (3) a review of the previous literature relevant to this approach.

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CHAPTER ONE

MODELLING AND THE COMPUTER

1. MODEL BUILDING.

Biological processes are normally highly complex in character. Whereas we can often treat systems in physics and chemistry in a direct mathematical manner, biology does not easily lend itself to such an analysis, possibly because most applied mathematics has been devised to provide a conceptual basis for understanding the workings of non-living systems. Certain branches of mathematics, for example differential equations, recurrence relations, and probability theory are of use in the analysis of certain aspects of biological systems, but it is only rarely that a full description of a dynamic living process can be given in the language of classical mathematics. The more complex a system, the more important it is to have a framework in which to give some kind of coherence to the information we have on the system - without such a framework we will not be able to handle the logical complexity needed to understand how the system works.

It is, therefore, important to know why there are additional problems in treating living organisms in the same way as inanimate processes. An insistence that the study of organisms requires factors additional to those of the physical sciences does not imply a dualistic or vitalist view of nature; living beings have been affected for some three billion years by historical processes which have had innumerable effects on them, most conspicuously on their genetic programs. Living systems also seem to organise themselves into individual units, generating

patterns in time and space which are recognisably individual and complete; the basic unit of these patterns is the cell, and the cellular multiplicity of most organisms gives additional complexity. It is this organisation, the stability which accompanies it, and the recording and passing on of the organisational history by self replication which results in behaviour most difficult to describe by formal mathematical symbolism. I hope to show in this thesis how the lack of appropriate mathematical formulations for describing some aspects of biological complexity may be surmounted.

Finding out more and more about how the component parts of a system are made up may tell us little about how the system as a whole works. An example often given is that of the watch: given a mass of springs and cogwheels, it would be difficult for someone unfamiliar with the workings of the watch to explain how it functions. A metallurgical examination of the structure of the cogwheels is clearly not of great value - it would be far better to try to piece the various parts of the watch together, and see how they work in conjunction with one another. The metallurgical study may be very interesting, but is of rather secondary importance. This difference between parts and whole has been discussed by several other authors. Weiss (1963) has used the argument "If a is indispensible for both b and c; b for both a and c, and c for both a and b, no pair of them could exist without the third member of the group ... in other words, a system of this kind can exist only as an entity or not at all". At the lowest levels, the properties of the whole system may not

be evident, and it is only be looking at the whole that the working can be seen.

Apter (1966) quotes the so-called 'levels of organisation' doctrine. "The living organism can only meaningfully be dealt with at a higher level than physics and chemistry, and at this level quite new phenomena emerge which are of the very essence of such self organising systems, and are missed at any lower level". Kacser and Burns (1967) have also discussed an approach of this kind, and warn of the dangers of the fine analysis of trivial phenomena, "a new methodological and conceptual approach is necessary if we are to make progress beyond the cataloguing of an even greater number of biochemical details".

If we have knowledge about the individual parts of a biological system, but no detailed information about the interrelation of the parts, then it may well help to build a model of the system. The word 'model' itself has been taken to mean different things in the past, and we will use the definition given by Kacser (1960). Kacser describes a model as "a statement, or series of statements in language. Models are therefore propositions, which may be either verbal or mathematical ... in which entities are related according to the rules of the particular language".

Kacser further separates models into two categories, heuristic and conceptual. A <u>heuristic model</u> is a model which is used as an aid to experimental work, and its predictions are usually directly testable by experiment. "It need not be a mathematical model, and it need not make quantitative statements,

nevertheless, it contains statements about the outcome of future experiments or observations, by means of which it can be tested for its applicability". We will see that the majority of the models to be discussed here fall into the heuristic category. Most biologists build heuristic models, indeed, experiments are usually designed as a result of such modelling. A model may be simply a means of classifying a group of related plants, a way of describing the interrelation of the components of a biosynthetic pathway, or a tentative verbal explanation of the working of a physiological process.

A conceptual model acts as scaffolding into which existing knowledge can be slotted, and an overall understanding of the system thus obtained. Conceptual models often correspond to 'theories', for example the 'theory of evolution'.

There is a third type of model which is also of importance in the work to be described in this thesis. Using the substitute system, studied for its own properties, insight can be gained into the workings of living systems which may seem to employ similar mechanisms to the model. Kacser emphasises that some overlapping may take place between the three main types of model. Other authors have also classified model types, including Apter (1966) and Apostel (1961), although differing nomenclature may be used. Apostel's 'Type H model' clearly corresponds to the heuristic model defined by Kacser.

Types of heuristic model.

There are several different categories into which heuristic models may be separated, and it may sometimes not be easy

to decide which is the most applicable. Goodwin (1970) has succinctly put the problem of deciding the one to use in any particular case. "The choice of (model) language, of formalism, lies before the investigator, and the only rule for this choice is the general one that the good butcher does the least cutting".

Mathematics itself can be regarded as an example of a model language, in certain instances, although we have already seen that its direct use is limited. In the cases in which it can be used, classical mathematical equations are constructed (or more often, adapted) and used to describe a process - an example of a 'mathematical adaptation' into biology is the use of statistical mechanics, first devised to predict the thermodynamic behaviour of gases, and recently used by Goodwin (1962) to formalize subcellular dynamic activities. In this type of model, and in the other heuristic models, adjustment in input 'parameters' to the model can often be used as a test to see if the model is behaving like the real system. The discrete, statistical or probabilistic model is another category. However, in this latter case, the elements of the system being studied are too complex to fit into the regular description given by a continuous model. It is with this situation, where we are constantly faced with trying to describe very complex situations, that the models described/are concerned. Statistical approximation is used to model determinate processes, where the 'determinate factor' is incompletely understood - this will be dealt with in detail in the following section.

here

Heuristic models need not be formal in the mathematical

sense, and in some cases a biological system can itself be used as a model, or else some physical analogue can be used. Many aspects of differentiation are studied by using organisms much simpler than those in which our interest may be centred - the interaction of two different tissue types may be studied in vitro using a millipore filter (Saxen 1971), or someone interested in cell/cell signalling in higher organisms may study the chemical impulses leading to aggegation in slime moulds (Cohen and Robertson, 1971). Electrical networks can be used as analogues to model nerve nets, and a membrane physiologist may use collodion films and silica gels as analogues of real membranes. Although these have the same functional properties, there is no physico-chemical equivalence. "In a sense, it is correct to say that the study of biological systems in terms of models proceeds by abstracting the physics and chemistry from the system, leaving behind a set of formal relations that may then be studied". (Rosen, 1968).

The simulation of deterministic behaviour.

As described by Tocher in his book 'The Art of Simulation' (1964), a parallel to the sort of complex situation we are confronted with in developmental biology was reached by the mathematicians von Neumann and Ulam, when faced with trying to find a solution for the diffusion equations. In diffusing systems, particles behave in a partly regular, partly irregular manner - by averaging over the particles, the random element was eliminated, and a deterministic description could be given.

This elimination of randomness in helping us to describe complex processes, where we could not hope to use a deterministic method is called the Monte-Carlo or random walk technique. The making of random choices fitting overall statistical patterns is also well suited to simulations of large cell populations. The majority of the models to be described here will be seen to use this methodology.

An essential factor in such simulations is the use of computers. As randomness is such an important factor, several repeat runs of a Monte-Carlo simulation have to be done in order to get a measure of the repeatability of a result. If the model is of any size at all, it will probably be too time consuming to entertain doing even one run by 'hand'. The use of a computer program as a realisation of a model will be defined here as a simulation. If we are faced, then, with a complex system that seems amenable to this kind of analysis, we can suggest the sorts of properties that such a model might possess.

The use of random numbers to represent 'deterministic behaviour' can be illustrated by the following example. A cell may divide in a particular direction because there is less physical resistance from the surrounding medium for this division direction. Now it may be that there will be 'random' fluctuations in physical resistance throughout the medium. In any one cell division, the division directions will not be random, but will be caused by the differential in resistance. As the cell division patterns overall will appear random, it does not matter if one of the cells divides to the left, or to the right, so

long as all the cells make the choice of division direction randomly. In other words, the use of randomness does not mean that the process being modelled is actually random <u>in vivo</u>, but that we cannot in practice work out the deterministic behaviour of the real system. This is a more practical statement of the 'levels of organisation' doctrine quoted on page 3. At the lower level, the determinism of the cell division directions is important, but when the whole population is considered, it is the overall behaviour patterns produced by the cells acting in concert that is more interesting.

The random choice can be made by deciding on the number of random choices to be made, and using a 'look-up table' to determine the outcome. If a certain random number is picked, then the look-up table decides the outcome, which part of the computer program is to be processed next. The flow chart in figure 1.1 shows how this can be accomplished.

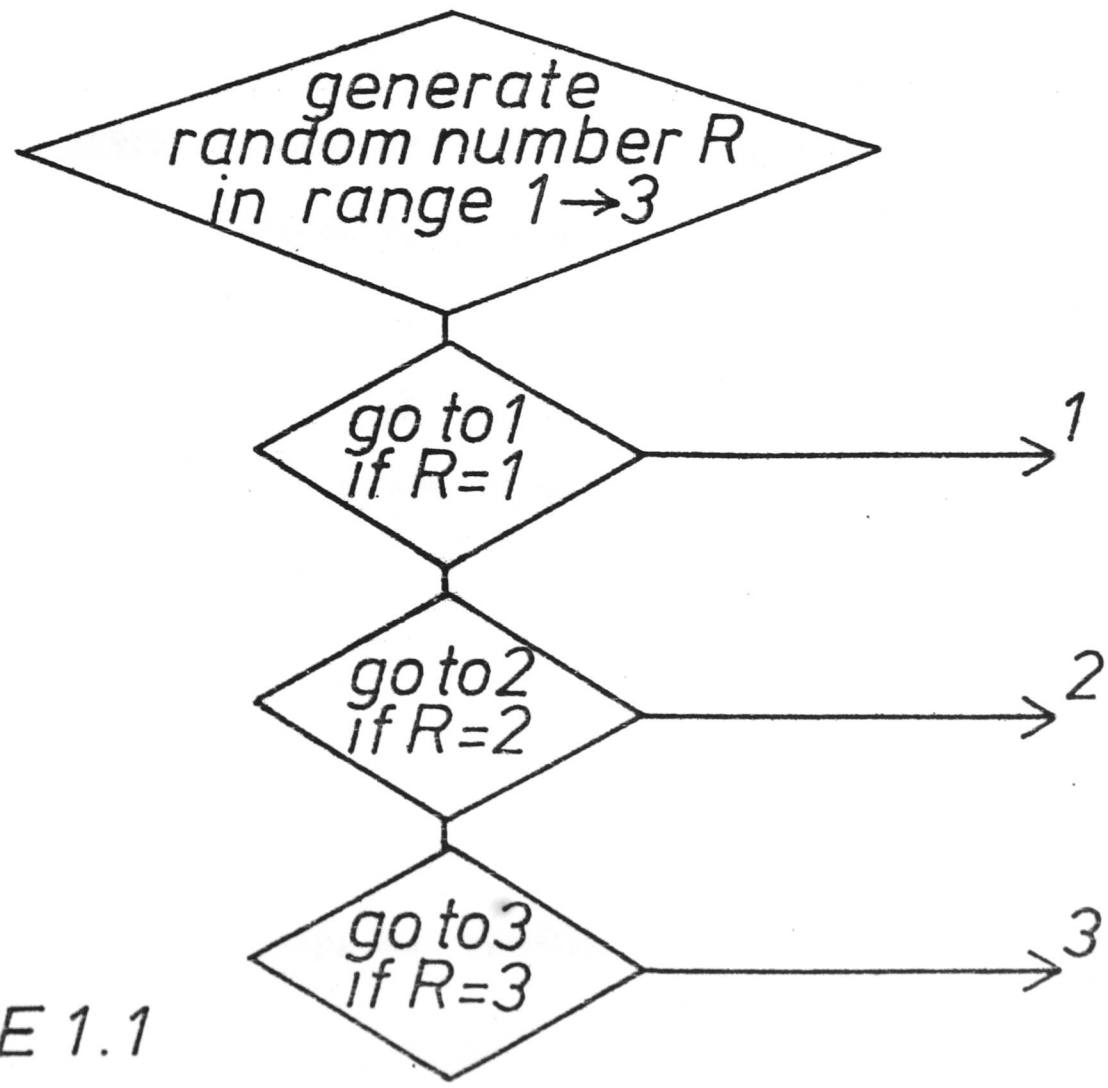

FIGURE 1.1

As the results obtained from a Monte-Carlo simulation are necessarily dependent on the sequence of random numbers generated, it is important to have a reliable source of random numbers. The computer itself can generate 'pseudo-random numbers', and this method is suitable for the simulations to be discussed here (most computer installations have 'house' software for producing random numbers, but see Jansson, 1963 or Tocher, Chapter 6 for methodologies). A pseudo-random number generator should produce numbers evenly distributed over the whole range, and with no correlation between adjacent terms. Pseudo-random numbers are generated in a cycle, but as the cycle length is of the order of millions, it should not affect the simulation.

Another common component of this type of simulation is the use of list structures. When modelling large numbers of units, like cells, it is often important to maintain and update information, whether it is about individual units or the whole population. We will see later how this is especially useful when maintaining lists of which cells have divided in a particular generation. This sort of operation can either be done using a specialized list processing language like LISP, or by using the facilities of a standard high level language. Although the latter is often slower, it can often be used with success. This is the approach I have used in the simulations to be described here.

The model simulation.

The design of a model to be run on a computer will take several stages. Firstly, the problem must be defined in a series

of clear statements. It must also be understood what the model is expected to achieve - it is little use preparing a large simulation with reams of computer output if the output tells nothing about the workings of the real system. The process of abstracting the most important factors of the system to use in the model is an art in itself, and often requires a great deal of practice. Secondly, the model is constructed from the abstracted statement. This stage involves making a flow chart of some kind, although the chart need not be as formal as a layout of the complete computer program with every individual instruction written in.

A flow chart is useful for several reasons. It enables communication of a computer program between users, or between user and programmer if the running of the program requires some specialist help. (It is difficult to understand someone elses program, especially when written in a different computer language). It also enables the programmer to see how much time is spent by the computer in processing various parts of the program. It may even be possible to give an estimate of the time a model will take to program. Although it is not easy to work out how long a program will take to develop and run, the number of branching instructions in the program give an idea of the 'logical complexity' involved. If a standard amount of programming time is associated with each branch instruction, based on past experience, an estimate of the total programming time can be obtained (Smith, 1968).

An important point to make at this juncture is that the mere formal construction of a computer model, before any computing

is done, can give insight into the workings of the real system, and so reduce the need to actually run the model on the computer. This is all to the good - few models are ends in themselves, and whether or not a full-scale computer model needs to be run really depends on the ability of the simulation to give meaningful information about the system being studied.

After the flow chart has been completed, the preliminary programming is begun. If the cost of computer time is no object, the modeller may be tempted to try to run the program before completely understanding it. This often causes much trouble, and it is only after completely and carefully analysing how the program will run that the computer should be approached. It is also important to know the capabilities of the machine to be used. Although cost may be no object, time may be, and computers vary significantly in their processing speeds and turn-round times. There are also additional considerations, such as the advantages of using multi-access systems, or specialized input and output facilities. Multi-access systems allow interaction between user and program whilst the program is running, and this can be very useful in some simulation work. Input and output will be dealt with in greater detail in the appendix.

The computer language used really depends on the requirements of the individual simulation. The program examples described here have used Edinburgh IMP, an ALGOL based high level language with no special simulation features. FORTRAN may be just as suitable in many instances. Various 'simulation languages' exist, but these are geared towards particular applications, and

would probably not be of great use in the sorts of simulations to be discussed here. Many high level language installations do have the basic facilities needed for simulation work including routines for providing pseudo-random numbers, and software for handling list structures.

2. CELL INTERACTIONS IN DEVELOPMENTAL BIOLOGY.

We have looked at the sorts of models that may be useful in studying developing systems. Now we must look at the application of these modelling techniques. The next chapter will discuss the various approaches made by other workers: this section will describe both the basic problems which we might use computers to tackle, and a criticism of a widely esteemed concept in modern embryology - 'positional information'.

As Waddington (1962) has discussed, developmental processes may be separated out into a number of groups. These groups have been described by Wolpert (1969) as molecular differentiation, spatial differentiation (pattern formation) and morphogenesis. Molecular differentiation is mainly concerned with subcellular organisation, and although we will mention it again briefly at the beginning of the next chapter, it is largely out of the scope of this thesis. Such unicellular organisms as Micrasterias, Acetabularia and Paramecium, and cells like neurons may show as much interior differentiation as some whole metazoans - a sponge, for example - and so subcellular processes are of extreme importance. However, the discrete solutions to the types of model to be described in the present work are more suitable for the analysis of phenomena further up the developmental hierarchy.

The simplification that spatial differences beget molecular differences begets morphogenesis has been previously thought close enough to the real situation for it to be used as a working basis for studies on development. To quote Wolpert (1969) "The spatial pattern of differentiation may be regarded as the process whereby a cell has its spatial position specified - positional information - and (that) it is this which can determine its molecular differentiation. Finally, with morphogenesis - moulding of form - the emphasis is on the forces bringing about changes in shape". And again, "It is important and convenient to distinguish between molecular differentiation, spatial differentiation and morphogenesis whilst recognising their interdependence".

Figure 1.2 summarises my abstraction of the essential elements of such a scheme.

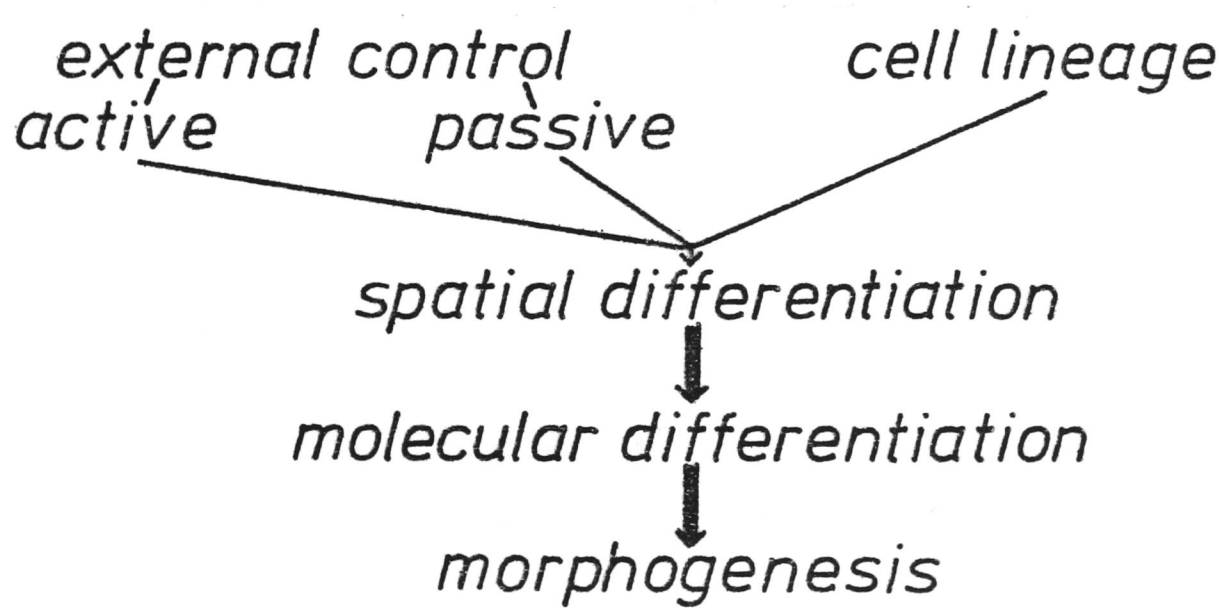

FIGURE 1.2 (see page 14 for explanation).

Spatial differentiation is the process by which regional differences in tissues, and local differences within these regions, are initiated. Molecular differentiation - clearly necessary before any changes in the cell can come about - is the result of responding to the differences imposed by the spatial vector. There are three main ways in which this can come about: external control, both active factors (like, gradients, the influence of biochemicals impinging on the cell from the exterior, or electrical impulses) and passive agents (crowding and pushing of cells producing deformation changes and influencing cell membrane receptors), also the effect of the past history of the cell line held in each cell.

The end effects of the 'differentiation' are the morphogenetic changes by which biological shapes are developmentally evolved. The potency of localized cell shape changes in development has been shown by Gustafson and Wolpert's (1967) work on gastrulation in the sea urchin. Indeed, the acquisition of spatial <u>similarities</u> may also give rise to shape changes, as Elsdale has shown with his 'inherently precise' fibroblast cultures (Elsdale, 1970).

Morphogenesis is often regarded, as in the simplified scheme of figure 1.2, to be the end product of molecular and spatial differentiation (viz. Wolpert's 1969 analysis). This kind of treatment does not make allowance for a possibly important factor - that 'morphogenesis' proceeds throughout the development of an organism and may well have an instrumental role in laying the foundations for the paths of future differentiations.

Great emphasis has recently been placed on the abstraction of 'positional information' as an important concept for understanding development. (Wolpert, 1969; 1971). It has been usual to consider active gradient mechanisms as central controlling factors by which the 'positional information' is set (see, for example, Lawrence, 1971; Wolpert, Clarke and Hornbruch, 1972; Postlethwaite and Schneiderman, 1973). An active gradient, for our purposes, is one which involves passage of some 'influence' along, for example, a line of cells. This influence imposes polarity on the system, and might be some biochemical factor or an electrical impulse.

There are many situations, however, where the development of pattern seems to arise as a direct result of cell/cell interactions. In such cases, a vectorial 'force' may be said to be present, but this may well not be best understood as a gradient. The development of cell compartments in Drosophila imaginal discs, discussed in Chapter five, may be a case in point. Here, the shape of cell clones is shown to be dependent on the physical forces produced by the surrounding constraints. Each cell clearly possesses 'positional information', and if removed from its constrained situation will show 'regulative development'.

When considering cases like these, it becomes difficult to remain within the 'positional information' code. A recent paper by Summerbell, Lewis and Wolpert (1972) shows this. Evidence is presented as to the mechanism by which limb bud outgrowth is controlled, and for the first time "autonomous" changes in positional value (my underline) with time, rather than

signals from 'far off' are proposed in this case as the specifiers of positional information.

Now, there is much evidence that cell divisions, and the control of these divisions, are important in development. In imaginal discs, for example, regionally patterned differences in mitotic activity have been observed (Becker, 1957), and the 'transdetermination' of disc structures after serial culture also illustrates the role of cell division in development. (Hadorn, 1966). Summerbell et al. propose that the specification by a graded signal is supplanted by a timing mechanism, in the setting up of the limb bud proximo-distal pattern. It is suggested that there is a "progress zone" immediately behind the growing limb bud tip, and that the character of groups of cells may be determined by the length of time they have spent in this labile tip region. By invoking cell division, the 'positional information doctrine' is stood on its head. I will illustrate this: Summerbell et al suggest that we look for a "biochemical correlate to the proposed changes in positional value in the progress zone". Any biochemical differences which may be found can either be interpreted as the result of changes in positional value (caused by other mechanisms), or may be regarded as the causes of the changes. (That is, the signal which the positional information concept assumes). Cause and effect are inextricably mixed up and any differences found are bound to reinforce the circularity of the hypothesis. This is exactly the pitfall of the positional information concept. It leads, like the earlier gradient theories of Dalcq and Child (Dalcq, 1938; Child, 1941) to searching for some solid biochemical,

when we may really be faced with a very different problem. The search for how a cell 'knows' biochemically its 'map reference' may be a complete red herring in some cases.

Cell division is a morphogenetic process. Addition of extra cells to a developing tissue gives rise to size, and possibly to shape changes. If cells are dividing in a particular way, say, constrained by surrounding tissues, then they may be pushed in particular directions. Being in the centre of a developing limb bud may provide a very different stimulus to a mesenchyme cell than being just under the surrounding epidermal layer. One could speculate, for example, that there are stress receptors in cells which cause differential responses only if certain stress patterns are set up in a tissue. The areas of cartilage condensation in the limb bud might fall into such a category.

Taking cases like these into consideration it is therefore appropriate to examine whether the concept of positional information is useful, or whether it is only a semantic device which conceals more than it reveals. Let us examine Wolpert's five propositions summing up the case for 'positional information' (Wolpert, 1969).

(1) "There are mechanisms whereby cells in a developing system may have their position specified with respect to one or more points in the system. When cells have their positional information specified with respect to the same set of points, this constitutes a field".

If these 'mechanisms' include every difference that can arise, both externally generated, by, say, a gradient, or internally generated by cell/cell interactions of a biochemical, physical or electrical nature, then this statement is nothing more than the truism that cells in a non-homogeneous milieu are situated in a non-homogeneous milieu. The problem of how this mechanism works is left completely vague, and yet, this is the central problem of differentiation.

(2) "Positional information largely determines, with respect to the cell genome and developmental history, the nature of the molecular differentiation that the cell will undergo. The general process whereby positional information leads to a particular cellular activity or molecular differentiation will be termed the interpretation of the positional information. The specification of positional information in general precedes and is independent of molecular differentiation."

If stochastic processes, (such as asynchrony of division, the X-chromosome inactivation in female mammals etc.) are disregarded as irrelevant to the problem of differentiation, since every cell has the same probability with respect to any of these processes, then the statement says no more than that any <u>differential</u> changes which cells in different parts of the tissue undergo must be attributed to the differences in the milieu already referred to. This must be true by definition.

(3) "Polarity may be defined in relation to the points with respect to which a cell's position is being specified: it is the direction in which positional information is specified or measured."

This is a partial restatement of (1), since the "polarity" is implied in the 'specification' of a cell's "position".

(4) "Positional information may be universal, that is the same mechanisms that specify positional information may be operative in different fields within the same organism, as well as in quite different genera or even phyla."

And it may not be.

(5) "The classical cases of pattern regulation whether in development or in regeneration, that is the ability of the system to form the pattern when parts are removed or added, and to show size invariance, as illustrated by the French Flag problem, are largely dependent on the ability of the cells to change their positional information in an appropriate manner and to be able to interpret this change."

This seems to say that cells have the property of interacting with their enviroment, and if this changes, the cells responses may change.

In summary, Wolpert's approach turns out to be a statement, in rather anthropomorphic language, of the orthodox view

that pre-existing heterogeneities, or heterogeneities generated within a growing and expanding system, affect the responses of cells, and their subsequent changes. The main task of identifying the nature of the heterogeneities, the levels of interactions which influence the subsequent history and the role of the genome (or particular genes) in these processes remains as obscure as before. It is not clear how Wolpertian language will help in solving these problems. The task at this stage seems to be to gain insight into the behaviour of 'cellular' systems which have specified and experimentally plausible properties. The emerging system properties will help us to assess the assumptions made in setting up such models.

Apter (1966) has proposed a list of 'axioms' of development which, he says, can be used as the basis for models of development. In brief, these argue that (a) the unit of development is the cell, and that all we need to know about it is its response to input and output. This fits well with the models to be described in this thesis. (b) Each cell starts off with the same set of basic instructions (i.e. the genome). (c) An organism develops through the self reproduction of the cells composing it. (d) Development depends to a large extent on communication between cells. (e) An organism controls the important aspects of its own development. Although it is useful to have such rules written down, Apter makes one seemingly major omission. He has no axiom to describe the acquisition of a particular differentiated state. We could add, therefore, (f) acquisition of a differentiated character by a cell is normally

irreversible in a developing system. It may proceed through a primary 'determination stage' where the cell has its fate fixed, but does not exhibit the final differentiated state.

To summarise the essential points raised in this section, and to put over the present authors point of view on the schematisation of developmental processes, figure 1.3 should be consulted, and compared with the previous figure. In figure 1.3, there is constant feedback between the extracellular effectors of development and the internal differentiation of the cell, so avoiding the linearity implicit in Wolpert's categorization.

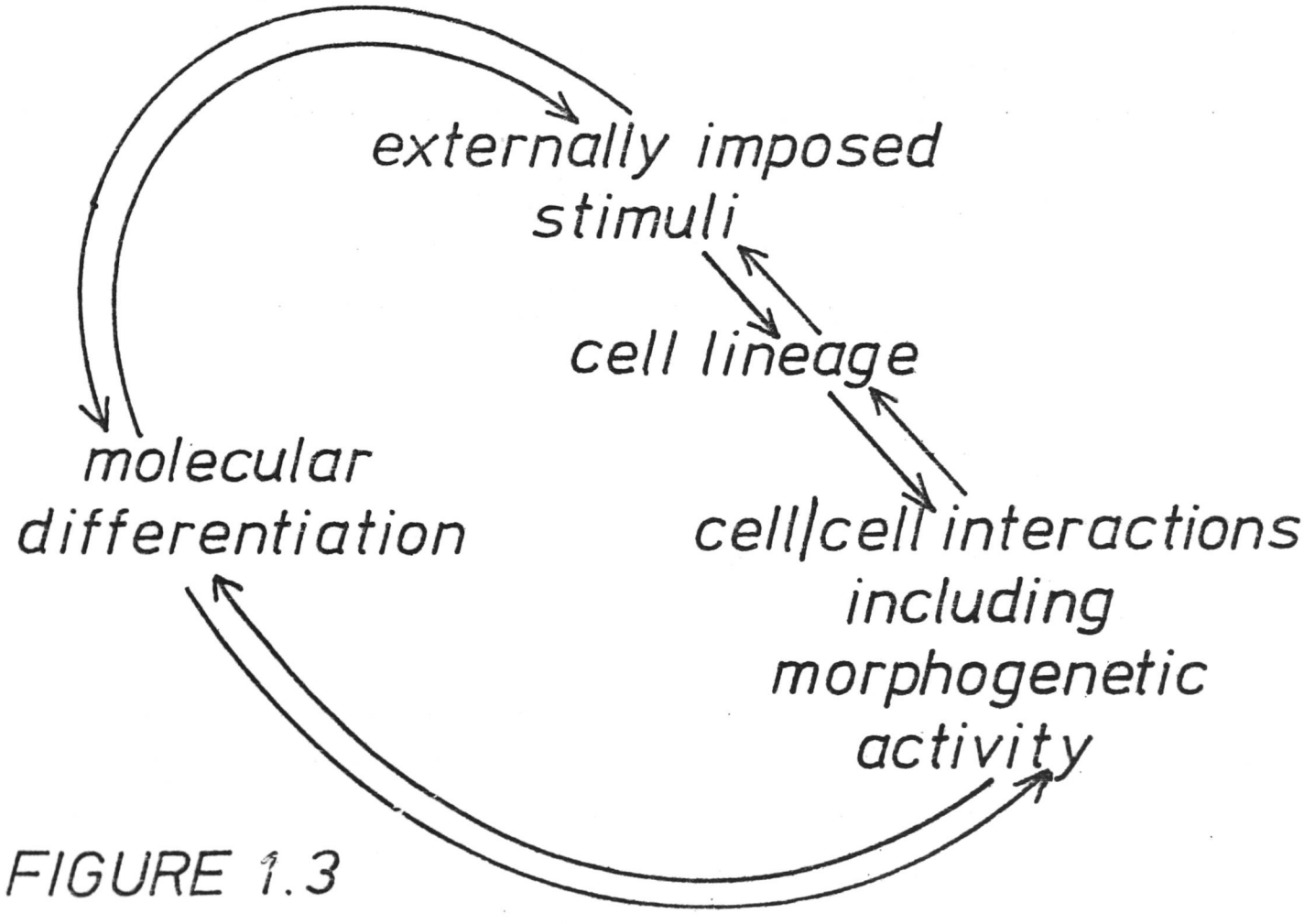

FIGURE 1.3

CHAPTER TWO

A REVIEW OF COMPUTER MODELS IN DEVELOPMENTAL BIOLOGY

INTRODUCTION.

There have been few previous reviews on computer models in developmental biology. Rosen (1968) deals with general cellular control processes and theoretical models made of them; he is not concerned with development *per se* although he mentions several of the examples to be quoted below. In a second paper (Rosen, 1972), Rosen reviews the theoretical aspects of morphogenesis. The review is largely limited, however, to an appraisal of 'self organisation' phenomena - molecules, viruses, and at a higher level, cellular re-aggregation. (This latter topic will be discussed later in the present chapter). Apart from Rosen's work, discussion of various models is to be found in Apter's book 'Cybernetics and Development' (1966). This book is now rather dated, (the majority of models to be described in the present chapter are post-1966), and as the title suggests, is biased towards the cybernetic approach: trying to show the validity of considering development within the reference points of cybernetics, Weiner's "Science of Control and Communication in the Animal and Machine" (Weiner, 1948).

A review of the kind attempted here may be written in several different ways. It is possible to describe the literature 'system by system', saying what models have been made of which biological systems. This does not work very well because there is no logical order in which to fit such a grouping: the

subjects chosen for simulation have been very diverse. It is also possible to list the various models historically, from the first models of Von Neumann and Eden in the 1950's onwards. As a third alternative, the simulations could be described from a computer viewpoint - the main feature of model X is the use of list processing, model Y, video techniques ...) Because all these methods have their own faults, it was decided to describe the models biological function by function. This leads to a classification in the following manner.

1. Subcellular models.
2. Cellular interactions. (Models using 'the cell' as the basic unit, corresponding roughly to 'morphogenetic models').
   (a) automata theoretic approaches
   (b) cell interaction models applied to specific systems
3. Models of pattern formation. (The patterns are externally imposed and normally do not use the cell as the basic unit).

1. SUBCELLULAR MODELS.

Little work has been done on the simulation of subcellular development, and most of the literature deals with continuous mathematical models of enzyme fluctuations in particular systems (see for example Ycas, Sugita and Bensam, 1965). A recent book 'Critical Variables in Differentiation' (Wright, 1973), expounds the basic creed of the enzyme modelling approach:

that we must find the biochemical controlling factors of a developmental pathway in order to be able to understand it. The 'critical variables', according to Wright, are invariably measurable concentrations of cellular components.

The work of Heinmets (Heinmets, 1966; 1970) uses the analog computer to process systems of differential equations representing 'enzyme synthesis', 'cellular growth' and 'reproduction'. Heinmets considers two systems in the former paper. The first is a single cell model with an enzyme system which can sense the cell's surroundings. The second model deals with the kinetics of cell interactions such as those leading to recognition and the formation of specific cell attachments in cell aggregates. This model uses some of the properties of the first model - 'sensing' exoenzyme elements associated with components transporting reaction products into the cell where their action can lead to induction or repression.

Both Heinmets and Wright believe that living processes are complicated but not complex, that these systems provide only problems of magnitude and not of concept; the computer simulation of non-linear problems, with appropriate variations of different parameter values, fed into the 'equations' describing the modelled system can provide the key to understanding the problems of cellular biology.

As the title of Heinmets book 'Quantitative Cellular Biology' (Heinmets, 1970) suggests, unravelling the complexities of cellular processes can only be done by differential equations if graphs of enzyme fluctuation against time are the key to all. They

may well not be.

Enzyme fluctuations normally occur as a direct result of gene control, and models have been proposed to simulate such cellular control processes. Stahl (1967) suggests that cellular control might be represented in terms of the logical manipulation of the nucleic DNA and RNA molecules. Stahl first proposed the idea of using a computer simulated Turing machine to do this* in 1963, and the subsequent development of list processing techniques† allowed modelling of a 'complex' cell system consisting of 46 genes that could demonstrate repeated 'self-reproduction' and 'differentiation'.

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* In a physical form, a Turing machine may be represented as a roll of paper tape with a scanner, as shown in figure two. A finite automaton such as we are considering, possesses a tape that has a finite length, and we define the machine in terms of Tape Symbols $S_0 \ldots . S_n$, and State Symbols $q_0 \ldots . q_n$. A quadruple is a collection of four symbols, specifying the program for the machine, and making the scanner move backwards and forwards on the tape in a prescribed manner. The tape is ruled into squares, each square having one Tape Symbol only.

e.g. $q_0 S_0 S_1 q_1$

This means 'when the machine is in state q_0 scanning square S_0, it must move into square S_1 and state q_1',

also $q_0 S_0 \longrightarrow q_1$

indicates that the machine in state q_0, square S_0, moves one

square to the right and takes up state q_1. A Turing machine can therefore be used in this way to handle numbers and functions as a decision procedure. It is, in fact, a primitive computing mechanism.

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† List processing languages have several important features.

(i) The program involves manipulating symbols that have non-numerical meaning.

(ii) The storage requirements need not be specified in advance; complex data structures are developed as the program is carried out.

(iii) The relationships among the elements of the data are restructured whilst the program is operating.

(iv) The program can be described at several levels of detail, and the problem is stated naturally in hierachical fashion.

List processing languages include IPL, LISP, and FLPL. The simple use of lists will be discussed in the next chapter.

DNA coding for each enzyme is represented by a four digit identification number and here numbers are held in a list. The RNA list contains complementary copies of the DNA, these copies being generated by an automaton acting as 'RNA polymerase'. An "enzyme list" contains the 'protein' equivalents (generated by the 'ribosome automaton' of the cell) corresponding to the RNA coding. Self reproduction involves a series of algorithms representing a build up of needed substrates. In Stahl's model,

the temporary cessation of activity of most genes, activation of 'DNA polymerase' and strand separation simulation to give two 'cells'. A special feature of the model is the 'differentiation controller' gene system which works as follows. Self reproduction continues providing the parent cells are still allowed to divide. Once a small colony of 'cells' has been formed, a build-up of by-product triggers the action of a differentiation initiator gene, stopping further reproduction, and causing the differentiation of cells into 'inner' and 'outer' types.

Stahl's list representations could theoretically be used to list any DNA, RNA or protein sequence. The substances used are arbitrarily simplified, in order to be simply handled by the computer program, but a similar methodology could be used to list, say, triplet codes. Stahl's procedure is discrete, and the main operations are integer threshold checks, additions, and subtractions. It is suggested (Stahl, 1967) that program subroutines could be built in to simulate various forms of enzyme kinetics to help avoid the inaccuracy problems inherent in discrete programs.

With Stahl's models we impinge on the field of automata theory, and this tends to pervade the whole literature of the computer simulation of development. It is not difficult to imagine the strings of polymer molecules as strings of symbols printed on the tape of a Turing machine, with the enzymes governing the rules of manipulation. This treatment of sub-cellular processes has been criticized by Goodwin (1970), who

thinks that the discrete treatment is too far removed from the real situation. The important points in any epigenetic process are the switching points, says Goodwin. Where cells must decide which of several alternate paths of differentiation to follow they must, in the language of computer programming, obey some sub-routine which comprises a form of conditional instruction. 'If condition A is satisfied, do B'. This suggests that if a cell works in the same way, it "computes its own state, looks at the DNA program, and then changes state accordingly". Goodwin argues that at the molecular level we will have problems with biochemical 'algorithms' because "biochemical switching is only analysable in terms of a (non-linear) interaction between the variables describing the state of the cell".

> "Let us suppose that a particular metabolite, y, is formed from two precursor metabolite molecules, u and v, the reaction being catalysed by an enzyme E_1. Suppose that a second metabolite, z, is formed from the precursors v and w, E_2 being the enzyme catalysing this reaction. If u, v, and E_1 are present simultaneously in sufficient concentration, but either w, or E_2 is absent, then y will be formed. If v, w, and E_2 are present in sufficient concentration, but either u or E_1 is absent, then z will be formed. These statements constitute the biochemical form of the conditional sub-routine." (Goodwin, 1970).

Goodwin stresses that, although this is clearly what happens, there is no "independent computation of cell state, reference to DNA for instructions, and subsequent change of state in accordance with these instructions". The role of the DNA is passive in that the state of the cell at any time is partly determined by the regulation of rates of molecular synthesis by DNA control.

It might be argued that there are some state changes which do appear to be directly mediated by DNA reference, for example, repression in bacteria. In such cases, the computer analogy may hold. Here, Goodwin's argument that "decisions are taken by the phenotype, whose properties are largely determined by the genotype" may not be true.

Lindenmayer (1971) has defended the position of the automata theorist in subcellular biology. His argument is as follows. It is quite possible to show the algorithmic operation of the DNA program. For example, the set of all the metabolites in the cell excluding active proteins and nucleic acids may be called C. At any moment in a given cell a combination of the elements of C (a subset) will exist. If we look at each gene and the enzyme it gives rise to as a transformation rule (transforming certain elements of C into other components) we can call the set of all these transformation rules P. At any time in a given cell a subset of P will be present. We can, therefore, assign a state consisting of subsets of P and C to the cell. The <u>inputs</u> and <u>outputs</u> consist of C fluctuations in and out of the cell. The <u>next state</u> depends on the cell's present state and the inputs

it receives. Lindenmayer states that the only real objection against these kinds of constructs is an objection against their discreteness. He stresses that the advantages of dealing with integer entities include conceptual and computational simplifications which may be necessary before we can understand these systems.

2. CELLULAR INTERACTIONS.

(a) Automata theoretic approaches.

The initial usage of automata theory in a biological context comes from the work of von Neumann. (von Neumann, 1966). Von Neumann's main interest was in self replication, and he worked on two models of self replicating systems. The first of these was the kinematic model, a hypothetical robotic representation of a self replicating system. It consisted of a central set of computing elements - switches and delays, controlling an artificial hand, or muscle-like element; cutting and fusing elements to make new parts; rigid elements out of which the other types were made, and sensing elements. The automaton 'sat' on a lake of parts, and made copies of itself in Turing machine-like fashion, by sensing and scavenging for parts.

The problems behind making a workable kinematic automaton would have been immense, and von Neumann soon switched to the two dimensional "cellular" automaton. In this way we are introduced to the two dimensional array, an essential part of many of the simulations to be described in this chapter. The notion of a cellular automaton is built up in the following way. We

begin with a cellular space, consisting of an infinite n-dimensional Euclidean space, together with a neighbourhood relation defined on this space. The neighbourhood relation gives the list of neighbours for each cell. There is also a discrete time base t = 1,2,3, ... for the system. Each cell has a finite list of states, and a rule which gives the state of a cell at time t+1 as a function of its own state and the states of its neighbours at time t. The list of states, together with the rule governing the state transition of a cell is called a transition function (corresponding to the 'transformation rule' of Lindenmayer).

Von Neumann devised, but never programmed, a cellular automaton of huge complexity - each cell possessed 29 distinct states. Codd (1971) has shown that this can be reduced to a programmable 8 states per cell, and other workers have also simplified von Neumann's original model. The uses of the cellular automaton lie mainly in programming theory. For example, a growing cellular automaton that is synchronously dividing into a number of replicates might be programmed to carry out a certain computation. If the replicates can simultaneously carry out such a computation, parallel computing may be possible. Other hopes for cellular automata are that they can be used to make their own computer software - 'growth' and 'repair' of automaton logic circuits might then be feasible.

This sort of approach is far from biology, and workers studying the problems of development starting from the basic definition of a cellular space tend to fall into two groups. In the next section we will deal with the simulations which use the

arrays and self reproduction ideas as a starting point. Other authors have tried to view intercellular developmental processes in automata theoretic terms. Arbib (1967) has probably done most to further the cause of the automaton in developmental biology. Arbib stresses that the problems of self-replication of interest to the automata theorist do not end with the "largely trivial" (Arbib, 1967) DNA→RNA and RNA→enzyme transductions. It seems much more important to find out how a complex multi celled automaton can grow from a single cell. Arbib admits that most work at the present time is on "ingenious programming of cellular arrays, rather than on weaving a rich texture of theorems". He introduces the 'Mark II Module Model' - each module is an automaton representing a cell. The 'biological program' exists as a string of instructions stored in each 'cell', and only a portion of the control string can be read by each individual cell. The change in activation of parts of the string is the model equivalent of 'differentiation', and increase in cell number corresponds to 'growth'.

Lindenmayer (1967) looked at a simple model situation. He viewed a string of cells ('a filamentous organism') as if they are units that can undergo changes of state under inputs received from their neighbours. The cells produce outputs as determined by their state and the input they receive. Cell division occurs by inserting two new cells into the filament to replace a cell of known state and input, all cells to the right of the divided cell move outwards one position. A set of 'look-up tables' were used to compute the 'next state' of each cell. For simplicity, two

present state	input 0	input 1
0	0	1
1	11	0

[a]

generation	env. input	growth
0	0	1
1	0	11
2	0	110
3	0	1101
4	0	110111

[b]

generation	env. input	growth
0	1	01
1	1	101
2	1	0101
3	1	101101
4	1	01010101

[c]

FIGURE 2.1
Lindenmayer's filament growth model

differentiated cell types were considered, and these were represented by the integers 0 and 1. The most elementary example of Lindenmayer's model in action is as follows.

Growth in the filament is from left to right, and at any time t, next states are computed by the previous state of the cell and its immediate left-hand neighbour ('input') at time t-1. The 'look-up table' is shown below. (Fig. 2.1a.) If the 'environmental input' (the constant left-hand column) is set at 0, and we start with a single 'seed' cell, the first few generations will read as in figure 2.1b. Changing the division instruction to give (0,1) progeny gives rise to a repeating banded pattern - fig. 2.1c. If the environmental stimulus is also changed, a banded configuration results.

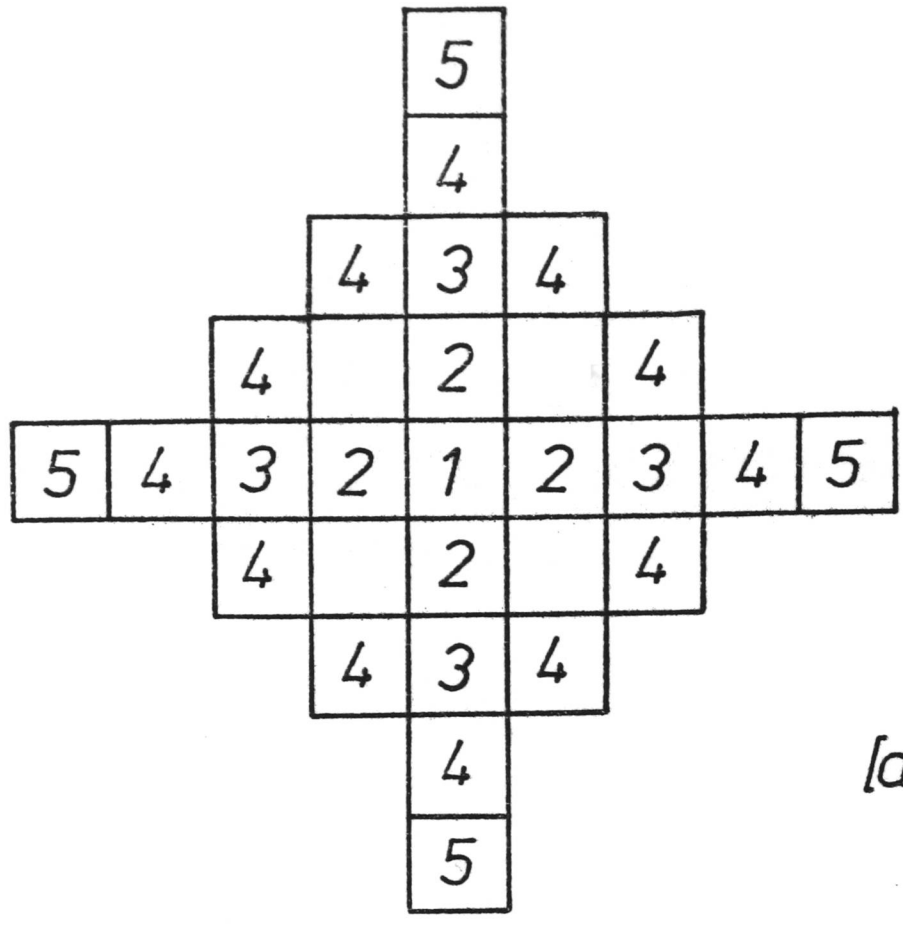
Lindenmayer decided that although such uni-directional input systems may be important in isolated situations (he quotes, for example, auxin travel in plants), in many more cases it is necessary to employ two directional input relationships. A further complication - the simulation of branching filaments - has also been discussed. (Lindenmayer, 1967).

These simple models have proved useful in the study of the link between automata and living organisms. Simulations of pattern formation in one dimension have been carried out using the heterocyst spacing pattern in Anabaena (Baker and Herman, 1972) as a model system, and Lindenmayer models (called L-systems by van Dalen, 1971) have been used to explain the workings of this spacing mechanism (Baker and Herman, 1973). Lindenmayer (1971) has also used the analogy of biological

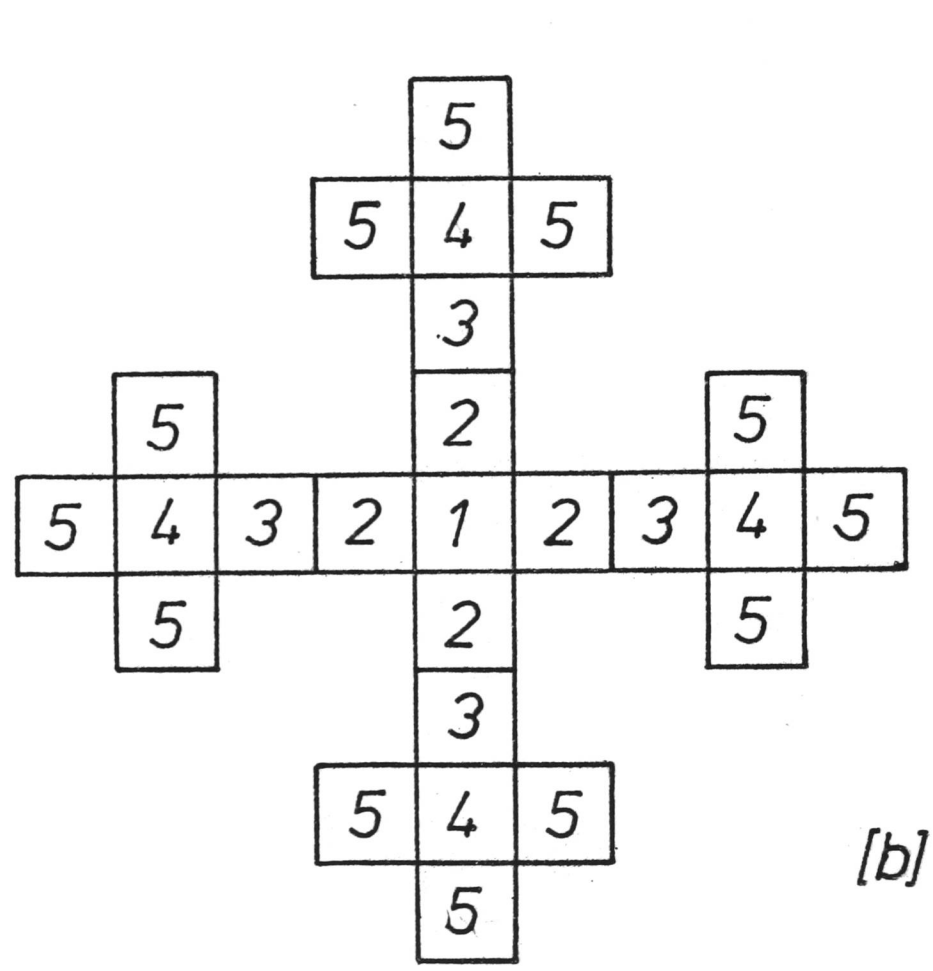
processes to language theory (see for example Bastin, 1968) to provide a possible 'language of development', complete with axioms, strings, and sets of productions; these ideas are based mainly on the L-system.

Among the models with no formal basis in automata theory are those of Ulam and of Eden. Eden's work has a grounding in statistics, and uses a two dimensional square lattice as a spatial framework (Eden, 1960). Each point in the lattice is a computer storage location that can hold a number representing a cell. If the model starts with a single cell in the centre of the lattice, and this cell divides so that its daughter cells are allocated randomly to surrounding array locations (with the proviso that cells can only divide when they have free edges and do not divide into diagonal array positions what sort of shape is produced, and how does direction-biased cell division affect the model?

Eden found using probability theory that it is possible for any colony shape with the same number of contiguous cells to be generated by the model. However, the probability of obtaining any particular shape decreases as the number of cells on the colonies edge (that is, cells with free surrounding array positions) increases. The result is therefore an 'average' circular colony, as seen during the growth of many bacterial colonies. If the probability of cells dividing along one axis of the lattice is increased, Eden showed that the axial ratio (maximum height/maximum length) of the colony is greater than the probability ratio of preferential division. If the probability



[a]



[b]

FIGURE 2.2
examples of patterns generated by Ulam

ratio was 10:1 for horizontal:vertical growth, the axial ratio is only about a third, much less than the 1/10 intuitively expected from the probability ratio.

Ulam was concerned more with the general problem of the complexity of patterns that could be produced from simple rules than with the probability of obtaining particular cellular configurations. Although his work is rather abstract (witness the patterns shown in his paper 'On some mathematical problems connected with patterns of growth of figures', 1962), it is included in this section because the generation of complex patterns is such a central problem in developmental biology. Like Eden, Ulam's models most usually employed the subdivision of a plane surface into squares, or cells, and 'rules' for the 'birth' and 'death' of each cell were specified. As Ulam devised a vast number of rules, two of his rules will suffice to illustrate the technique.

We start in the first generation with one occupied square and define the growth rule; given a number of occupied squares in the nth generation, the squares of the nth + 1 generation will be all those adjacent to only one square of the nth generation. Figure 2.2a shows how growth appears after 5 generations, using a four neighbour space. If the rule is extended to include inhibition by cells which touch diagonally, Figure 2.2b is produced after 5 generations.

Two other workers have also considered models of this type, and have drawn parallels between the simple model that uses recursively applied rules and developing systems. Maruyama

introduces the concept of 'deviation amplification' (Maruyama, 1963). Cybernetics is a science of self-regulation and equilibration but, says Maruyama, little notice has been taken of systems which generate change rather than uniformity. Ulam's models fall into this 'neglected' scheme. Maruyama (1962) introduces the idea that this sort of complex pattern formation is very relevant to genetics - the genes do not need to contain the whole amount of information necessary to produce the adult organism - all they need to specify are some simple rules of interaction between the component parts of the embryo. "Each part of the embryo does not contain the information for all the details of what it is to become. The parts generate the information by interaction, i.e. each part receives some information, not from other parts but rather from its relationship to other parts." The amount of information held by the egg and its relevance to information theory (Shannan and Weaver, 1950) has been the subject of various analyses, for example by Apter and Wolpert (1965) and by Raven (1964).

Gordon (1966) presents a model that simulates the growth of a spiral generated by stochastic means. Using a two dimensional array, he defines two types of cells I and S. Growth of these cells occurs into non-diagonal surrounding array positions. Only one S cell, called the 'leader', may divide at any time, and the daughter cell becomes the new 'leader'. I cells grow randomly, whilst S cells divide into the left-hand site (relative to the vector from the cell the dividing cell grew from). If this site is not empty then forward and right-hand sites are

tested in turn and used if available. If the cell is totally trapped, then the first unsurrounded S cell nearest to the growing point divides.

Cell division occurs sequentially and a parameter r is defined such that the probability that the next cell to divide is an I cell is rP_S, where P_S is the probability that it will be the S 'leader' cell. All I cel s have an equal probability of dividing, if they have any free sites available.

Gordon shows how variation of r can give spirals with varying structures. If r = 0.4, for example, the spiral winds itself into a cavity. If r = 0.1, very wide spiral turns are exhibited, whilst r = 0.25 gives an equally spaced Archimedean spiral. Gordon also presents the basis for a 'general model of development'. This would include an organism represented by an ensemble of units (cells) all capable of making decisions. For each cell, these decisions are based on the state of the cell and its environment. (A restatement of the antomata theoretic definition of a cellular space). The internal state of each cell would include its state of differentiation, a 'memory', and perhaps an internal clock.

Gordon suggests that such a general model may be used to 'evolve' computer organisms. "Let an organism grow according to a set of rules R. R may be regarded as a vector whose components are a set of independent 'growth constraints'. Choose at random n slight variations from R, $R + \Delta R_i$, i = 1 ... n, and grow the corresponding organisms. Those which show

hints of some form or pattern which is aesthetically pleasing or biologically important are selected, and the process is repeated." Gordon also quotes Braverman and Schrandt (see below) who see future research in this field as "the form of a continuous dialogue between question and suggestion, arrived at through computer generations, and biological experiments and observations answering and proving these."

(b) Cell interaction models applied to specific systems.

Raven (1968) and Raven and Bezem (1972) provide a link between the models that have already been discussed in this section, and models more applied to specific developmental situations. Raven criticises the approach used by the automatists - the models designed by these authors show only a remote and superficial resemblance to any real developing organism. Raven suggests that this is because the automatists are not developmental biologists, and automata theory is not exactly equivalent to the processes that go on is embryogenesis. Having said this, he goes on to describe the morphogenesis of the larval head pattern in Limnaea, the pond snail, using very automata theoretic terms for the basis of his model (Raven, 1968). In this paper, he puts forward a set of simple rules for generating the snail head pattern. These rules are based on cell lineage: the internal state of each cell is dependent on that of its parent, and on the direction of the division by which it is produced. "The cell, so to speak, 'remembers' the previous history of the cell line of which it forms a part."

Raven uses a notation to specify the developmental

history of each cell, so

$$e(v, s, d_1) \longrightarrow D_1$$

means that the cell in question has developed from the ovum (e), and has undergone 3 divisions, one dorsoventral, one sinistrodextral, and one in the animal/vegetal plane. The cell is at present undergoing another animal/vegetal division.

It is suggested that early morphogenesis in the molluscs is very dependent on the placing and direction of cleavage furrows (Raven, 1966), and that for this reason the specifications in the model are quite valid. Raven also suggests that at this early stage cell interactions are of little importance, and that it is the inbuilt characters of each cell which determine the pattern of development.

Raven and Bezem (1971) use the rules formulated above to produce a computer model of embryonic development of the Lymnaea egg, the model simulating growth from the 4 cell to 64 cell stages. Apart from the assumptions of the 'paper and pencil' model, it was necessary to provide a geometrical base for the computer program, and this was done by representing each cell as a sphere. All cleavage planes pass through a fixed point on the egg axis, producing two daughter spheres. By varying parameters such as the angle of division, the position of the axial fixed point, and the proportion in which each cleaving cell is divided, a '64 cell embryo' was produced, and the printouts (done on a graph plotter) looked very similar to those previously shown in the actual analysis of the Lymnaea egg (Verdonk, 1968). Raven and Bezem take this to indicate that Raven's original

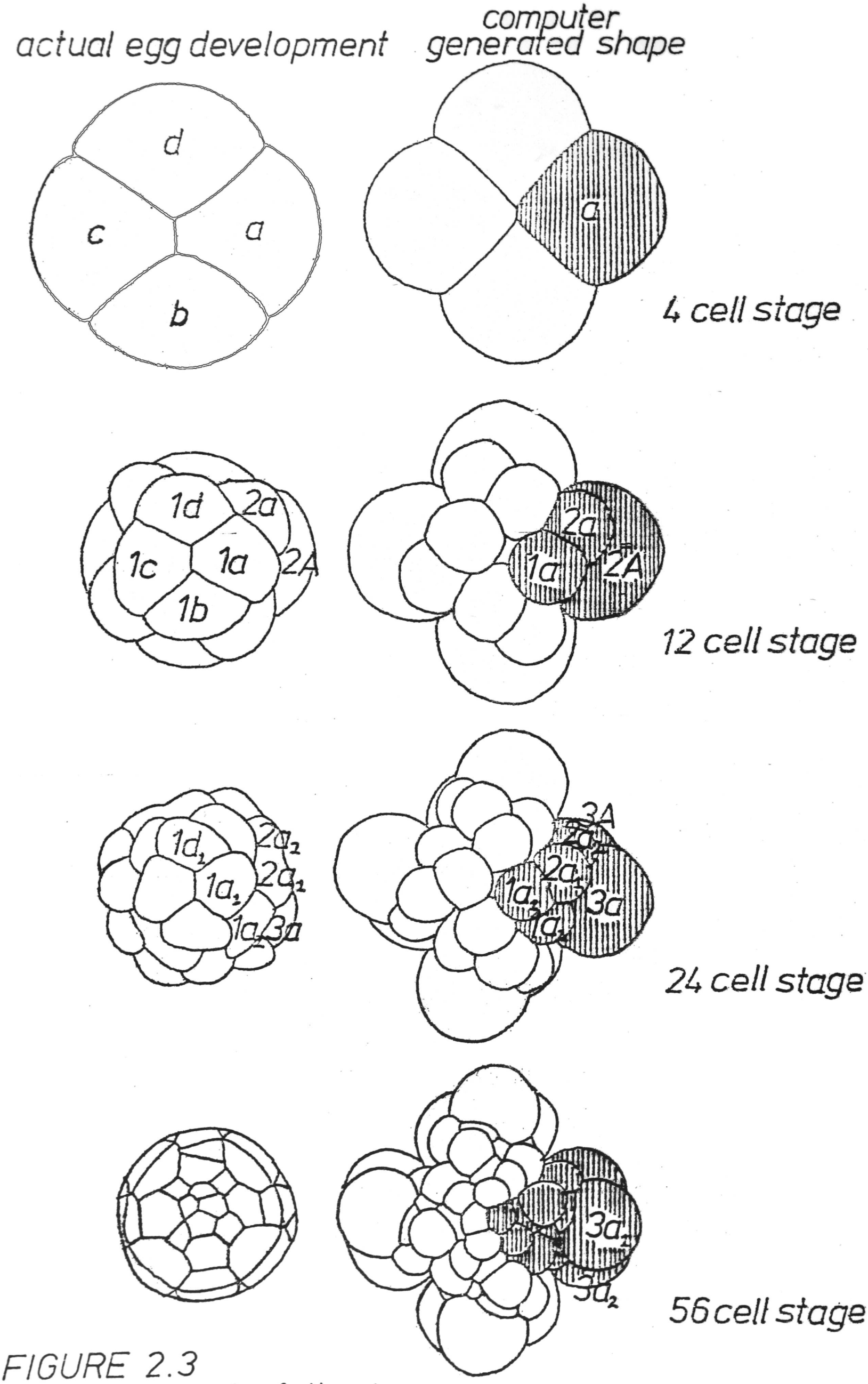


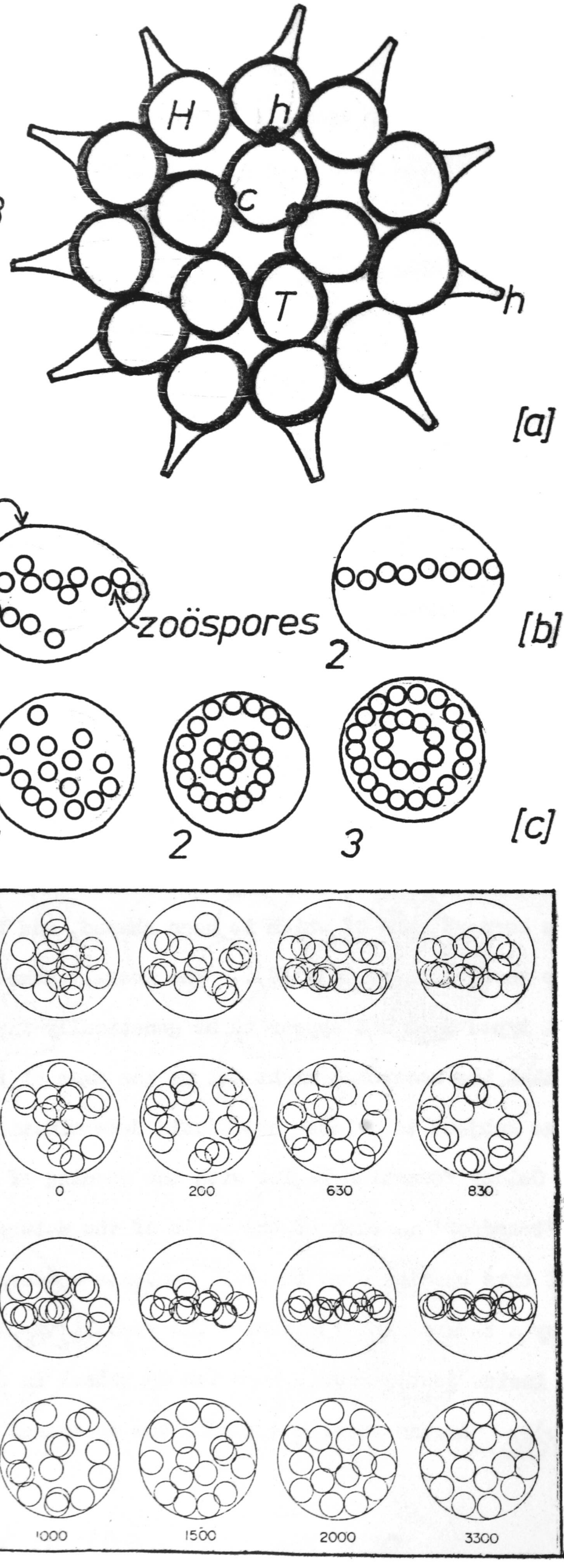
FIGURE 2.3
Development of the Lymnaea egg
(Raven and Bezem, 1971)

thesis, that early morphogenesis in molluscs is determined largely from within the cells themselves, may well be true. Figure 2.3 shows a series of comparisons of simulation with drawings from the actual developing Lymnaea egg. As Raven and Bezem themselves conclude, it is surprising that the simulation, only based on the information inherent in each cell, and devoid of any 'cell interaction properties' can give a close fit to the development of the real Lymnaea egg.

Honda (1973) has also used a geometric method this time to show how a particular living system may use the principles expounded by Ulam, Maruyama and Gordon in its development. Honda has analysed growing colonies of the coenobial green alga Pediastrum biwae, colonies of this organism being normally flat with 4, 8, 16, 32 or 64 cells per colony, Fig. 2.4a. There are two types of cells in a colony of Pediastrum; horn cells situated peripherally (H), and triangular cells (T). Horn cells have three corners, one of which is horn shaped, and triangular cells have three corners only (C). The overall number of each of these cell types does not appear to be genetically fixed: Honda suggests that the zoospores might all be the same at first, and then become subject to a later stochastic determination.

Colony formation begins with the opening of a semi-circular 'trapdoor' in each of the cells of the mature coenobium, and out of this opening come the cell contents - zoospores enclosed by a transparent vesicle. The vesicle expands, and the zoospores inside jostle round, becoming spherical in shape, Fig. 2.4(b)1. As the vesicle expands, the zoospores aggregate

FIGURE 2.4
model of
zoöspore
alignment-
Honda, 1973



into a plane, one zoospore in thickness (at this time the vesicle is almost spherical) Fig. 2.4(b)2. The zoospores link to form strings and finally become several concentric circles; before motility ceases, the zoospores change shape from spherical to conical. The vesicle finally disappears, and 'differentiation' into the mature colony is seen.

Honda suggests that the zoospores have three sites on their equators, one H - site, which can grow into a horn, and two C - sites which cannot do so, and grow into connection points between zoospores. If there are other cells nearby which prevent the growth of the H - site, this site will grow into the third corner, and the cell will become a triangular cell, otherwise it will become a horn cell.

One of the central mechanisms in the morphogenesis of the Pediastrum colony is the arrangement of the zoospores into a plane. Davis (1964) suggested that the vesicle was lens shaped, and so constrained the underlying spores. This observation has been countered by Hawkins and Leedale (1971) who saw normal colony formation within vesicles up to 3 X the width of the zoospores.

Honda has produced a computer model of zoospore alignment. The model makes two assumptions. Firstly, the H and C - sites all have an affinity with each other. Secondly, the zoospores move so that they keep their equators at about the same angle. For example, if one zoospore collides with another, the two move together for a distance, maintaining the contact. The computer 'zoospores' move inside a simulated large spherical surface, and the initial co-ordinates and equatorial angles of the

zoospores are determined by generating pseudo-random numbers. At each step of the program a zoospore is chosen and it moves forward for a set distance. If it hits another zoospore, the equators become parallel to each other (this simulates the affinity of the H and C sites), and the new orientation direction of the equators is chosen by averaging the directional cosiness of the original cells. If either of the spheres collides with a third sphere or the wall of the surrounding sphere during movement, the movement of the two zoospores ceases, and the next program step begins by random selection of another zoospore. Parameters R (radius of the containing vessel) and r (length of movement path) are varied. The problem of printing out a three dimensional picture was overcome, as with Raven's model, by using 'z-x' and 'x-y' plane projections to give a view from above and across the equator respectively (Fig. 2.4d). With 16 'cells' and using the rules described above, it was found that the cells arrange themselves into a flat plate after about 3300 moves - the model, therefore, suggests that the arrangement of zoospores into a plane is, like the early morphogenesis of Lymnaea, due to the properties of the individual units, and not to any overall external 'control system'. It still remains to be shown, however, that the angular buffering of the zoospore equators is actually shown by the living system.

Braverman and Schrandt (1967) have also produced a model based on the 'complex shapes produced by simple rules' theme, in this case during colony development of the polymorphic hydroid Podocoryne carnea. This organism normally colonizes the exterior

of the shell of the hermit crab, but a single hydranth removed from the shell will form a new colony on a microscope slide immersed in sea water, making it easy to see the shape of the colony that develops. The growth of the colony proceeds at a constant rate, and the ratio of hydranth number to stolon length is kept constant (Braverman, 1963). The distance between hydranths is therefore approximately constant, and follows a roughly normal distribution. Growth ensues until sexual zooids are formed, and then the rate of hydranth production slows down. Further singularities in growth are seen as the colony increases in size. As the colony of Podocoryne is limited to the plane of the slide, it is well suited to a two dimensional representation of colony growth on the computer. Braverman and Schrandt modelled this growth by using a two dimensional array with the elements in a square relationship to each other (see next chapter p. 67). Stolon growth took place by allowing adjacent lattice points to be 'added' to the existing stolon - the end of each stolon was tested in turn and a probability set that new growth would occur: this probability was calculated from the existing amounts of stolon and numbers of hydranths within a set distance of the growth point. Whenever a set number of new stolon lengths had been added, a new branch point was initiated.

By using rules like these, it was shown that the overall patterns of differentiation seen in the hydroid colony could be generated. As the computer rules used were based on randomness, it was suggested that the development of the Podocoryne colony also has a large stochastic component.

A similar model of hyphal or filamentous growth in two dimensions has been used by Cohen (1965), although this model does not claim to model any living process. It uses the same type of 'local density' growth rule for adding on new lengths of filament, and by varying the growth rules, very different growth patterns are produced.

Williams and Bjerknes (1972) have produced a model simulating abnormal clone spread from a 'tumour' through epithelial layers. The cell division activity of the epithelium is centred in the basal layer, Leblond et al (1959) showing that both daughter cells of a division remain in this layer, and displace neighbouring cells. Bjerknes and Iverson (1968) have suggested that tumours are induced by an 'initiator cell' giving rise to a fast-dividing clone which takes over the entire basal layer, pushing the non-tumorous cells out of this layer. To find out how fast the tumorous cells would have to divide to do this, Williams and Bjerknes simulated the situation by using a two dimensional array with the elements packed in a hexagonal arrangement. As the displacement of neighbouring cells of the same type would have no effect on the basal layer, it was only necessary to 'divide' cells at the tumour boundary. The computer maintained a list of boundary positions and processed cells at this boundary. Williams and Bjerknes defined a term, the 'carcinogenic advantage' which is the ratio of speed of division of tumour cells to normal cells. If N is the total number of abnormal cells, and n the number of abnormal cells at the tumour border, then

$$dN/dt = (k - 1)n.$$

Williams and Bjerknes suggest that a tumour starting from one cell dividing with K = 1.1 will take 13 years to reach a size of 2mm, on the basis of their simulations (or 1.33 years with K = 2).

Jorgen Carlsson (personal communication, 1972) has also experimented with computer models of tumour growth. In this case, a different question was asked. What is the effect of certain doses of radiation on tumours? Carlsson used a three dimensional array representation of a tumour, with the irradiation parameters (distance of beam, strength and sensitivity of cells) built into the program. The central problem with this work seems to have been the difficulty of visualizing three dimensional data with line printer output.

Models have also been constructed of cell division systems by making family trees of the growing cell population, (Valleron and Frindel, 1973). The model system used by those authors includes full data on the length of the various parts of the cell cycle, and can be used to simulate autoradiographic labelling kinetics. Our main interest in this system is the method of maintaining information on the age and state of each cell in the population. This is done by employing a two dimensional array Y(J,K)* where Y(J,K) is the time elapsed between time 0 at which the simulation was started and the end of the Kth phase of cell J.

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* If g generations are simulated, the dimensions of the array will be

$$J = 2^g - 1; \quad \text{and} \quad K = 4$$

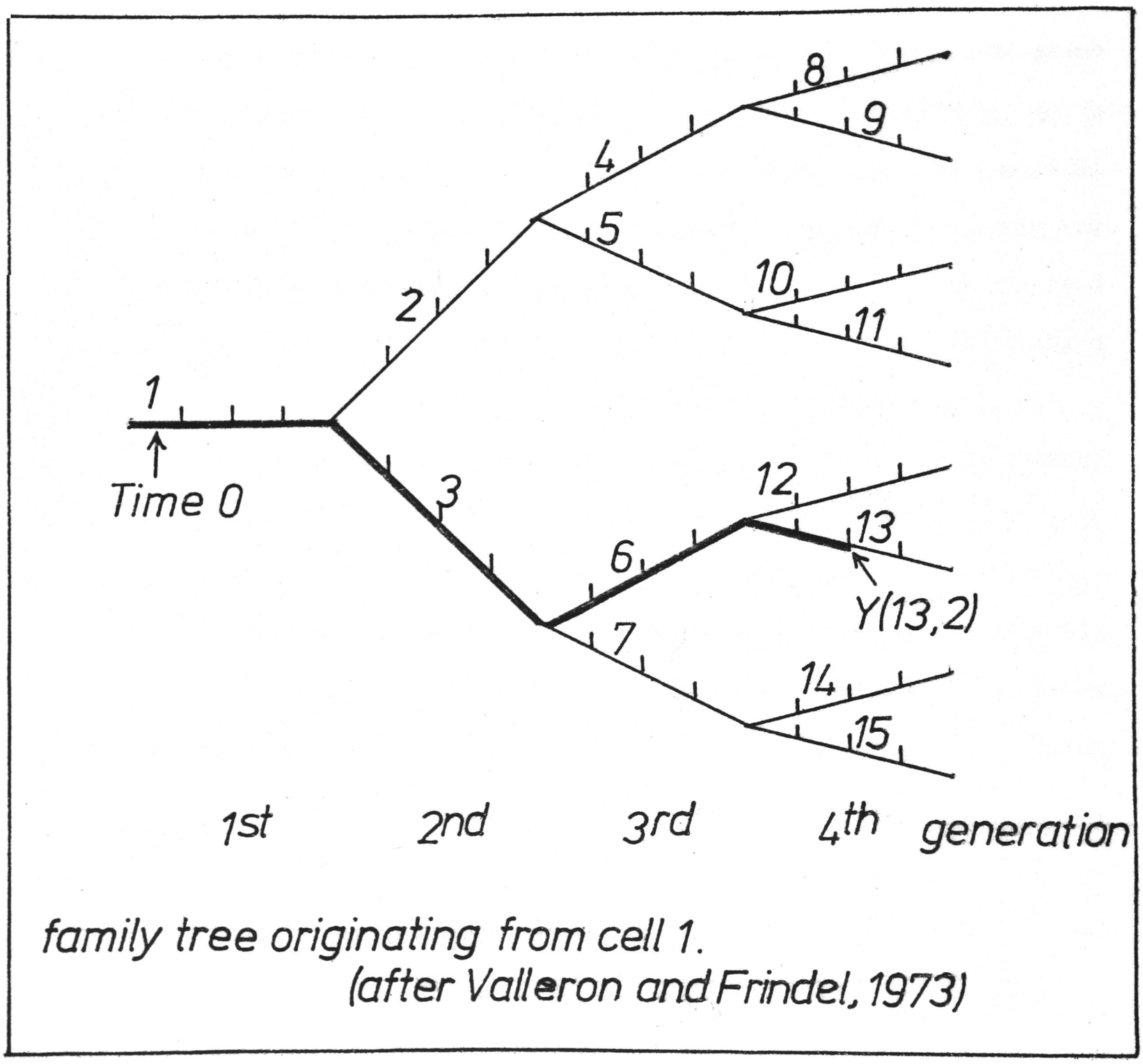
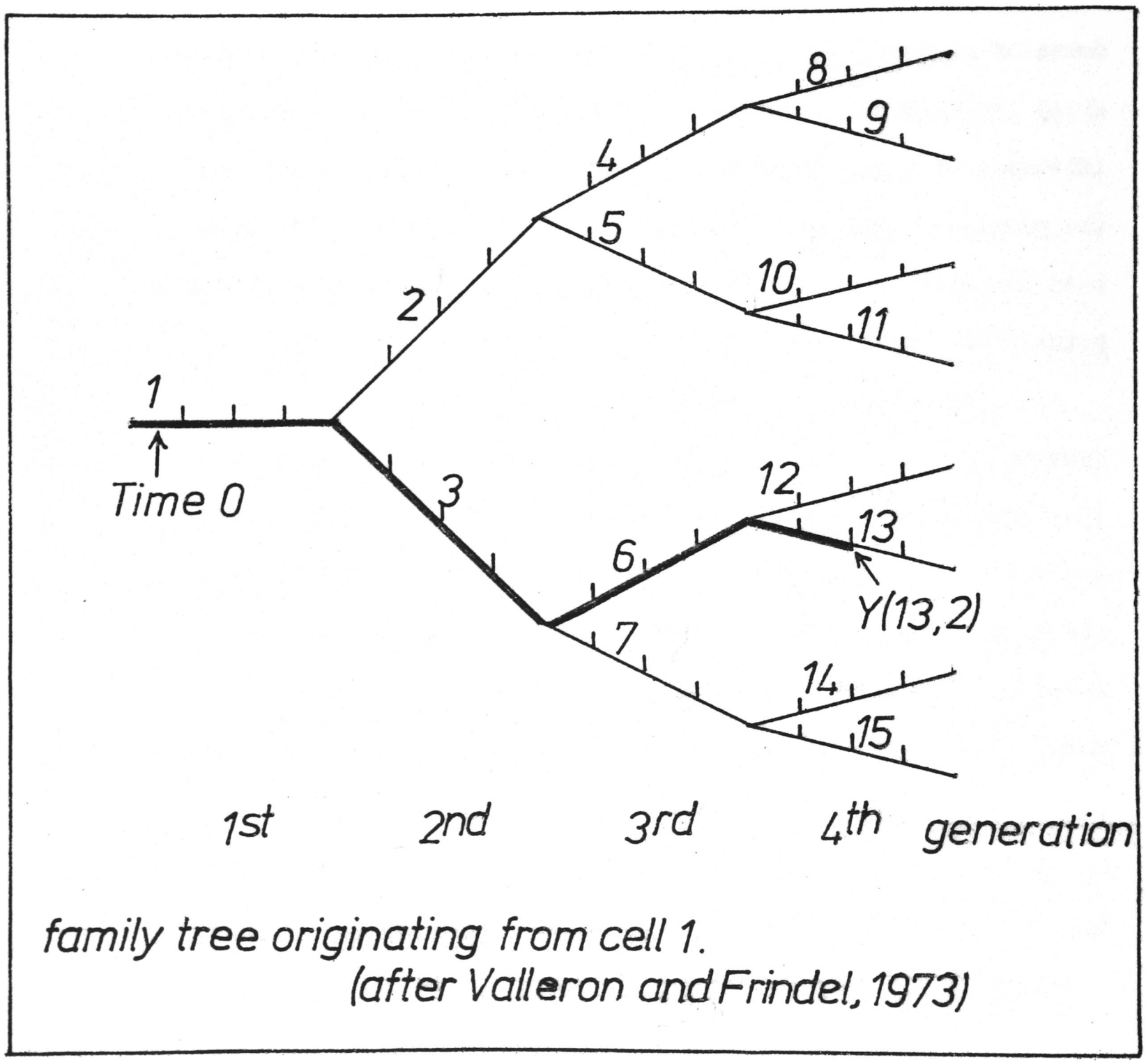


FIGURE 2.5 Each branch represents a cell, with vertical strokes marking-off each period in each cell cycle. The thicker line shows the lineage of a particular cell (13) ending the S (= 2nd) phase of its cycle. This cell can therefore be referenced Y(13,2).

K represents the phases G_1=1, S =2, G_2 =3, M =4 and the times for these phases can be set as parameters. The procedure for identifying cells is carried out by saying that if J is the number associated with a specific cell, and J_1 the number associated with the mother of that cell, then the relationship J_1 = INT(J/2) (INT means 'integer part of' the number in brackets) holds. Figure 2.5 shows how this nomenclature works in practice.

There has been controversy over the mechanism of growth in the limb bud for many years, mainly centering around the importance and role of the apical ectodermal ridge on morphogenesis. The role of the ectodermal ridge could be looked upon as a paradigm expressing the views of the two schools of thought regarding pattern formation and morphogenesis - is the role of the ridge to passively mould the underlying mesoderm (Amprino and Camosso, 1965), or are diffusible substances, like the hypothesised 'ectodermal ridge maintenance factor', and a second signal diffusing back to the mesoderm involved? (Zwilling, 1970).

Many research groups support the latter theory, with local differences as to its exact working. We have already seen in chapter one how Wolpert's group, whilst admitting that cell division differentials seem to be of importance in limb bud development, suggest searching for a "biochemical correlate" (Summerbell et al., 1972), which might control the division differences. Unfortunately, it is easier to speculate about ways of controlling complex processes than to admit that we might not yet be able to understand the ways in which they work - not



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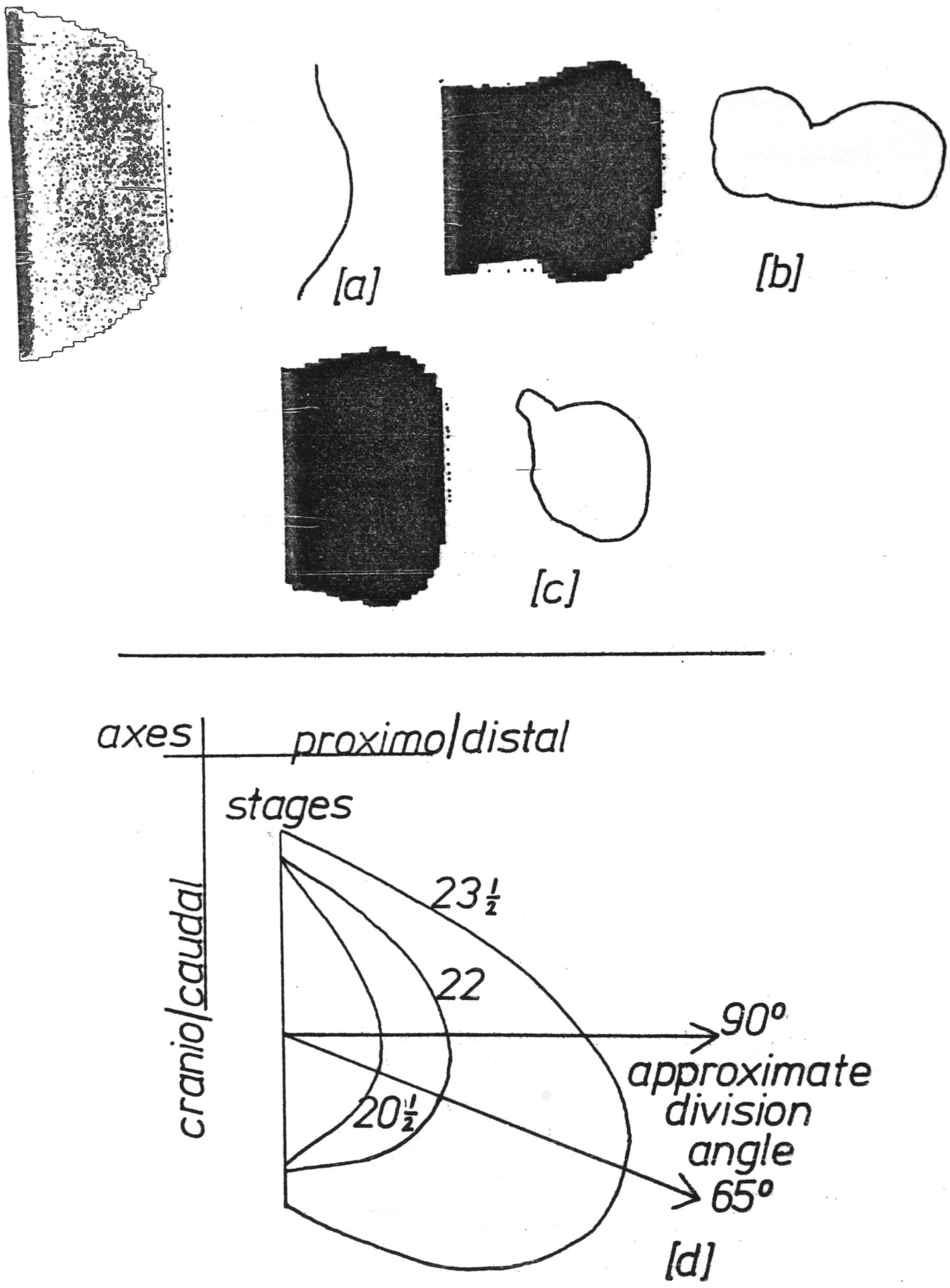

FIGURE 2.6 Computer simulation of limb bud development. Top: redrawn from Ede and Law (1969). Black shapes are computer generated 'limbs', and outlines represent actual limb bud shapes. (a) Simple cell proliferation in computer model gives approximation to early stage of limb bud formation. (b) Distalward cell movement + mitotic gradient in the computer model gives 'paddle' shape of later growth. (c) No distalward movement in computer model gives shape approximating to the talpid mutant. Bottom: Mitolo's (1971) model. Limb bud outline at three stages of the equivalent 'real' limb bud growth. A division angle of 65° to the baseline was used.

being able to <u>understand</u> something is very different to not <u>knowing</u> how something works.

Two computer models of limb bud growth appear in the literature. The first is by Ede and Law (1969). Ede's model uses a two dimensional array, with array elements containing the usual numeric codes representing 'cell' or 'medium'. Starting from a baseline of cells, cell proliferation (with each daughter cell being placed in the nearest available site to its parent) produces a mound of cells representing the early limb bud. (Figure 2.6a). The model includes provision for 'gradients' of cell proliferation in the proximal distal direction, and also allows the cells to 'move' distalwards by a specifiable number of array locations in order to test the importance of cell mobility during development. If the distalward gradient is introduced into the model, a broadening of the apex of the 'limb bud' is seen, but the shape is still very squat. Elongation of the stem region can be introduced by allowing a slight distalward cell movement. If this movement is combined with the mitotic gradient, and the distalward movement is reduced slightly at the apex, a shape looking rather like that of the late limb bud is seen. (figure 2.6b). If the distalward movement is omitted, a shape looking like the <u>talpid</u> mutant is seen (figure 2.6c). As Ede has already shown (Ede and Agerbak, 1968) that <u>talpid</u> limb mesenchyme cells are less motile than normal cells, the model seems to fit the possible causal factor behind the mutant.

Ede's model makes no attempt to simulate the influence of ectoderm (all cells in the model are of the same type). The

division algorithm used would only work with one cell type - any form of clonal growth requires 'space clearing' next to the parent cell (see next chapter p. 77). It also restricts itself to two dimensions.

Mitolo's model (Mitolo, 1970, 1971a, 1971b) also uses a one cell type system, and places great emphasis on the relative angles to the 'baseline' at which the cells of the growing limb bud divide. An array format is also used to give a spatial co-ordinate system. An axial direction of growth from the baseline is chosen, together with a standard angular deviation from this direction: this deviation governs the variation from the mean division angle that any one cell can make when dividing.

Mitolo also includes a proximal-distal gradient of cell division in the model, and concludes that a gradient roughly in the proportion 1:2 with 2 fold more divisions in the distal region, and with the growth angle inclined at 60° to the base and with $= 40^{\circ}$ is the recipe for an accurate - looking two dimensional limb bud (figure 2.6d)

In his latest paper (Mitolo, 1973), this simple model is taken further. Mitolo has found that his own model, like Ede's, could simulate development of the talpid mutant. In this case, the rule was simply that cells should divide completely at random, and possess no specific division direction. This appears at first sight contrary to Ede's model, but not so: distalward cell movement was programmed in the Ede model by making the dividing cells place their offspring in the nearest available distalward array position, and this is equivalent to division with axial direction

90° to the baseline. Mitolo's normal limbbuds show similar growth to Ede's, except for the fact that the angle between baseline and outgrowth direction is 60°, and the broadening of the apex is missing. To summarise, no distalward movement = no defined angle of growth = talpid mutant. This work shows that a lot of thought should be given to what a model actually represents. Ede's model preceeded that of Mitolo, but Mitolo shows basically the same thing. Thus, there may be many formal representations of a system, but they may all be interpreted in terms of the same mechanism.

Cell aggregation has been studied by a number of groups of workers. The initial work was by Goel and his co-workers (Goel et al., 1970; Leith and Goel, 1971), who set out to show that the 'work of adhesion' hypothesis of Steinberg (1963) held for a two dimensional simulation. Steinberg's hypothesis was as follows. If a mixture of two cell types a and b is allowed to reaggregate after separation into individual cells, the way in which the reaggregation occurs is dependent on the work of adhesion possessed by each of the cell types in relation to its neighbours. The work of adhesion for such a two cell type system could be represented W_{aa}, W_{ab}, and W_{bb}. Work of adhesion is an analogy with the attractions between molecules in thermodynamics. Steinberg proposed a set of rules for reaggregation dependent on the relative strengths of the attractant forces: if $W_{ab} \geq \frac{W_{bb} + W_{aa}}{2}$ then there will be intermixing of the two cell types: if the opposite,

$W_{ab} < \frac{W_{aa} + W_{bb}}{2}$ is true, then segregation will occur. If $W_{aa} = W_{ab}$, then complete sorting out into two separate populations will take place. If $W_{bb} < W_{aa}$, than what Leith and Goel (1971) call an 'onion' configuration will occur, with the a cells forming a ball inside a surrounding spherical mass of b cells. This occurs because the system will tend towards a minimum surface area, and a minimum free energy per unit of this area. If the thermodynamic reasoning is correct, then only having all the cells of type b on the free periphery of the sphere will give the minimum configuration.

This hypothesis is attractive, and neatly fits Steinberg's (1963) experimental results. However, an alternative mechanism has been proposed by Curtis (1971). Curtis thinks that reaggregation is a kind of 'repair' system: when cells are disaggregated, they lose their surface attractions, and reaggregation is merely a function by which the several different types of cell reacquire their normal cell surface properties. Curtis has carried out experiments on sponge cells (1969) in which the time at which the different types of sponge cells were reaggregated was varied. It was shown that the final configurations of the cell types differed when the time parameter was changed.

There is also a third, although out of favour, mechanism proposed to explain reaggregation phenomena, due to Holtfreter and Townes (1956). Whereas, Steinberg and Curtis both invoke rather novel phenomena dependent solely on cellular interactions, Holtfreter suggests that specific chemical messengers

are involved. Both Steinberg and Curtis have concentrated on simpler cell aggregation systems than Holtfreter's disaggregated embryos - Curtis on sponge cells and Steinberg on simple combinations of embryonic cell types. No evidence supporting Holtfreter's mechanism has been forthcoming as yet.

Goel et al., (1970) devised a two dimensional model simulating the cell aggregation process. Their model uses a four neighbour cellular space, and each contact edge between two cells is assigned a binding affinity, λ. λ is equivalent to W in Steinberg's terminology, thus λ_{aa}, λ_{ab}, and λ_{bb} may be assigned. An 'E function', roughly analogous to the surface free energy of Steinberg is defined as the number of edges multiplied by their λ values in a given pattern. The E function for a pattern is, therefore,

$$E = \lambda_{bb} N_{bb} + \lambda_{aa} N_{aa} + \lambda_{ab} N_{ab}.$$

Using this system, together with 'rules' for movement of cells, a two dimensional equivalent of a realization of Steinberg's hypothesis may be tested. From Steinberg's work, if

$$\lambda_{bb} < \lambda_{ab} \quad \tfrac{1}{2}(\lambda_{aa} + \lambda_{bb}) < \lambda_{aa}$$

then the only stable pattern will be when N_{aa} and N_{bb} are at a minimum, and this will only occur if the a cells are packed inside the b cells: this is the 'onion pattern'. The validity of the model can therefore be tested to see if 'onions' can be produced. In the computer model, λ_{aa} was made to = 1, and the other affinities equalled zero: A 20 x 20 lattice was used, and border effects avoided by placing a two cell width 'no mans land' around the grid. The a cells are all scanned once during each time step,

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and each cell tries to maximise N_{aa}. This is done by calculating the increase $\triangle N_{aa}$ that would occur by movement to any of the neighbouring locations. If N_{aa} can be increased, swapping of the picked cell and the cell in the chosen array position occurs. Goel's first paper (Goel et al., 1970) concludes that the simple application of rules of this type is unable to give the 'onion' configuration. The situation appeared to be rescued by a second paper (Leith and Goel, 1971) in which onion configurations were obtained. Leith and Goel investigated a large number of varying motility rules, and they concluded that the production of 'onions' depends on the ability of cells to 'feel' the presence of other cells several cell diameters away. Not only this - the cells can also move over two or three cell diameters by 'hopping', and subsequent swapping with the cell in the required site. These rules have been criticized by Antonelli et al (1973). Antonelli, in fact, throws doubt on the validity of simulating the onion pattern at all stating that Elton and Tickle (1971) have shown that central clumps may be more dispersed, and that ther may have been detection errors in looking at sorting out phenomena.

Antonelli's own model (Antonelli, Rogers and Willard, 1973) uses a hexagonal array. (Inaccurately stating that Goel's group dealt solely with square arrays - Leith and Goel (1971) actually also give examples of the use of their model with a hexagonal array). As evidence that hexagonal arrays are the most suitable for simulating two dimensional cell masses, Fejes Toth (1964, quoted by Antonelli) has constructed a theorem stating that convex planar 'cells' in maximal contact are circular in shape, have a fixed radius, and are arranged in a hexagonal 'beehive' pattern.

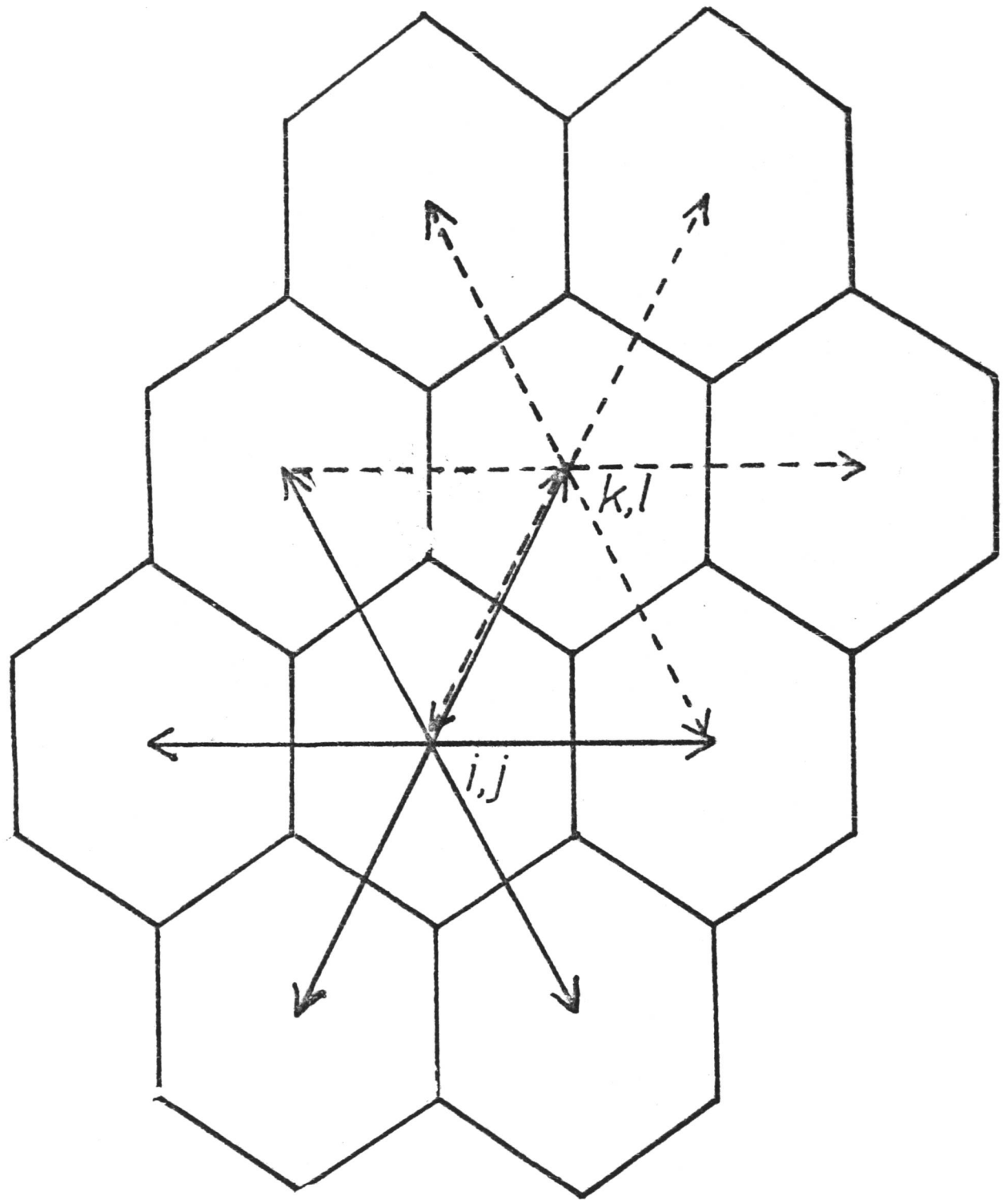


FIGURE 2.7
Antonelli's i,j rosette system

The cell with co-ordinates i,j is chosen, and it 'inspects' its six neighbours. On picking a neighbour (with co-ordinates k,l), the neighbour immediately signals whether it is of the same type as the cell to move or not. If it is not, it adds up the number of cells of its own type in the surrounding six sites, and the number of cells of its own type that it would have if it moved to the i,j position. The change Δ_{kl} = kl(after) - kl (before) is then computed.

Antonelli's model is founded on the exchange principle, which says that any neighbouring cells may change position only if the interchange is beneficial to both. Finding out whether the move will be beneficial is done by what Antonelli terms the 'i,j rosette system' (figure 2.7). The cell with coordinates i,j is chosen, and it 'inspects' its six neighbours - on picking a neighbour (with co-ordinates k,l) the neighbour immediately signals whether it is of the same type as the cell to move. If it is not, it adds up the number of cells of its own type in the surrounding six sites, and the number of cells of its own type that it would have if it moved to the i,j position. The change,

$$\Delta_{kl} = kl(\text{after}) - kl\ (\text{before})$$

is then computed, where k,l are the coordinates of the neighbour.

i,j is also computed for a change with this neighbour site.

The maximal move is chosen when all six neighbouring sites have been processed, and if none of the sites fulfil the requirement

$$\Delta_{ij} + \Delta_{kl} \geq 0$$

no move is made. If the sum = 0, the move is a 'random move' with no benefit to either of the cells, but this type of move helps the assortment of the cells. Antonelli's model is simulated on a 40 x 40 hexagonal grid. As with Leith and Goel, $\lambda_{aa} = 1$, and $\lambda_{bb} = 0$.

In the results shown, no large central clumps are seen. This seems to indicate that the central clumping is a feature of the extended 'swapping' allowed in the Leith and Goel model.

Gordon et al., (1972) have investigated a less discrete model, involving the idea that cell sorting is carried out by the

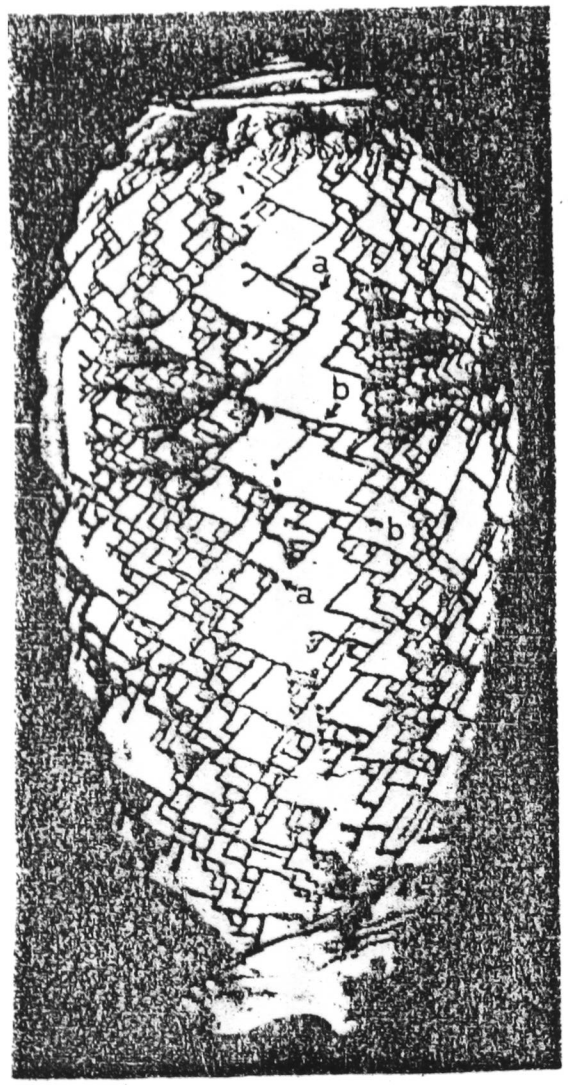
tension at interfaces between cells, and is resisted by 'tissue viscosities'. An analogy is drawn between this process and the kinetics of the break-up of an unstable emulsion between two immiscible liquids. As Goel is a co-author on this paper, it seems that his original array bound model may have been abandoned.

The kinetics of cell aggregation have also been studied by a Russian group led by A.V. Vasiliev. (Vasiliev, Pyatetskiy-Shapiro and Radvogin, 1972). The simulation is very similar to that of Leith and Goel, and also invokes the long range forces criticized in the latter model.

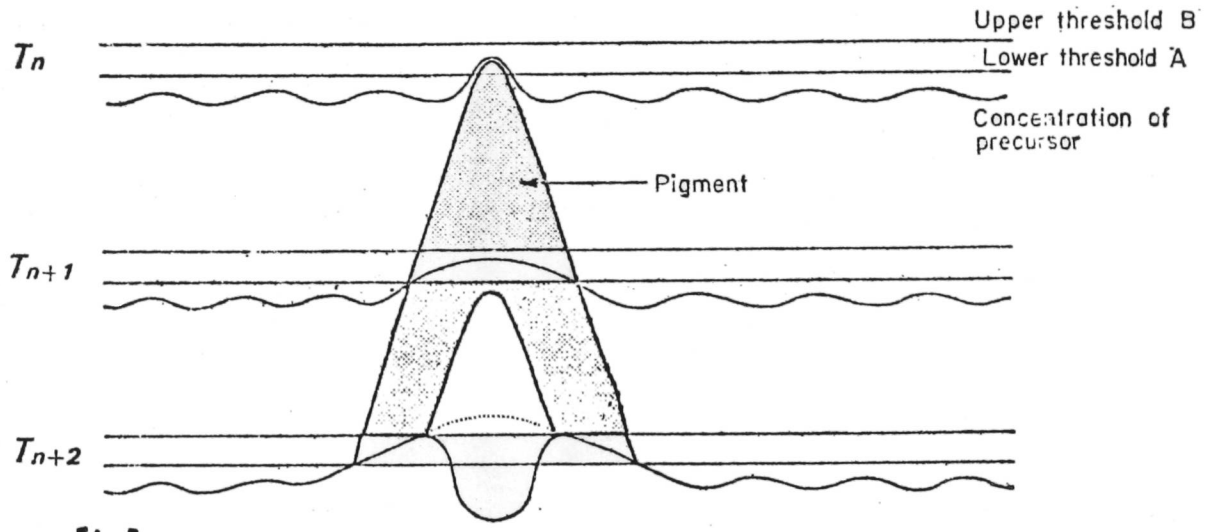
To summarize the findings from these models, it seems that the 'onions' initially hypothesised by Steinberg may be due to some other factor than simple differential stickyness of two cell types. Antonelli's group, using local exchange rules only, were unable to produce concentric aggregates. Antonelli thinks that the geometric configurations taken up by aggregating cell masses are not as easy to understand as Steinberg led us to believe in his original (1964) work, and that some non-random starting effects may be necessary to produce 'onions'.

3. PATTERN FORMATION MODELS.

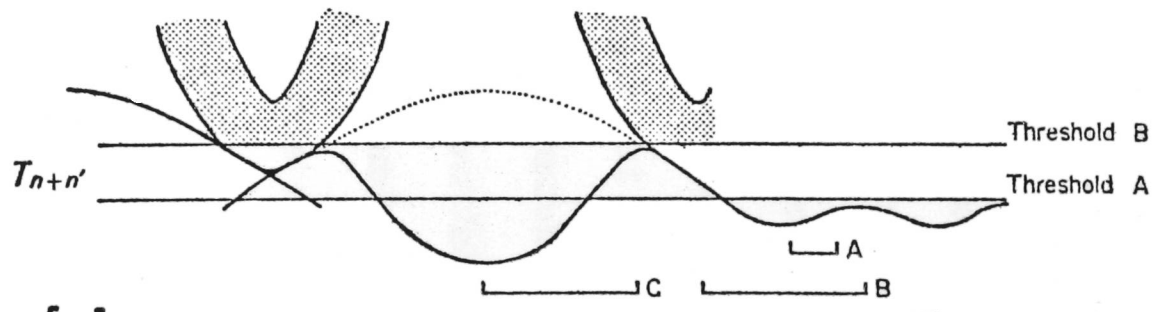
There are a number of models which fit into this category. Waddington and Cowe (1971) have simulated the formation of the pigmentation pattern on the shell Oliva porphyria (figure 2.8a). The growth of the molluscan shell occurs by constant production of shell material at the growth edge, and the pigmentation is laid down with the shell at this edge. The pigment is produced in



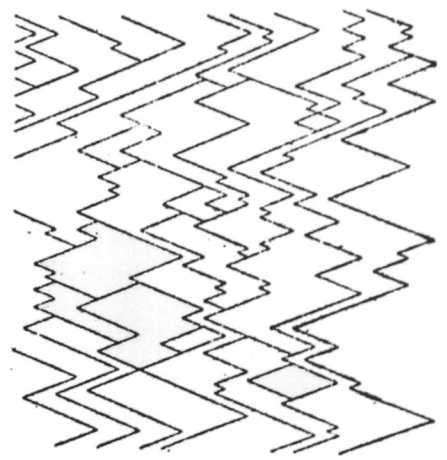
[a]



[b] Diagram of three stages of the hypothetical pigment forming system. At time T_n the concentration of precursor has fluctuated above the lower threshold at one point and pigment deposition begins there. At time T_{n+1} the concentration at the initiation point has risen, and the precursor diffuses laterally, so that the area of pigment deposition becomes broader. By time T_{n+2} precursor concentration in the central region has risen above the upper threshold, and pigment deposition has ceased there, leaving two diverging lines of pigment.



[c] On the left, the meeting of two lines of pigment deposition, showing the two concentrations of precursor which will be added together. On the right; if a stochastic fluctuation in precursor concentration occurred over a region A, this could lead to the appearance of a new initiation point for pigment deposition; but if the fluctuation always had a longer wavelength a fluctuation as in B or C, the initiation point would be brought on to the existing line of pigment.



[d]

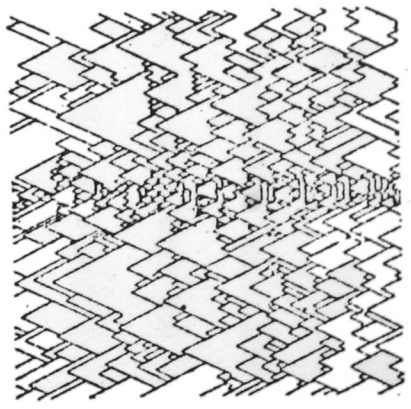


FIGURE 2.8
Simulation of pattern on shell of Oliva porphyria
(figures reproduced from Waddington & Cowe, 1969)

continuous lines: Waddington and Cowe have suggested that pigment initiation points arise stochastically, and from each initiation point diverge two lines of pigment. These lines are gradually pushed away from the mantle edge as it grows, giving a pattern looking like a network of 'tent-like' shapes (the common name of the shell is the 'tent olive'). When two lines of pigment intersect, pigment deposition ceases: it is suggested by Waddington and Cowe that the mechanism explaining this pattern formation process is based on the relative concentration of pigment precursors in the growing edge of the shell. The concentration of pigment precursor is hypothesised as being randomly distributed throughout the mantle edge. If the precursor concentration rises above a certain value at a point, pigment deposition starts. At a later increment in time, lateral diffusion of the precursor broadens the area of pigment deposition. Another essential feature of the model is an upper threshold above which precursor is destroyed or reduced in level; if this concentration is reached in the centre of the high concentration region which is laying down pigment, deposition will stop, and two diverging lines will be formed. If two of these lines meet, the addition of their precursor concentrations carries both above the upper threshold, and deposition ceases in both lines. (Figure 2.8b and c).

The computer simulation of this model used a video display screen to enable the operator to watch the pattern being built up (see appendix for details of video techniques). The use of video enabled user/computer interaction, so that the operator could vary the parameters until a realistic looking

pattern was produced. The program was based on an array, and particularly important was the right-hand most column of this array, which acted as the 'mantle edge' depositing 'lines of pigment' - these lines subsequently moved leftwards across the array as the column of 'deposited shall material' was pushed across the array. The irregular variations in precursor concentration were simulated using a pseudo-random number generator (see last chapter, page 9). For each initiation point, two lines of 'pigment' diverged at an angle which could be varied by changing a program parameter. It was found that the first attempts at the simulation failed because large white triangular areas, a characteristic feature of the shell pattern, were missing. The angles on the actual shell also pointed in a 'growth direction', i.e., the angle of each joining pair was canted over to left or right, the whole pattern 'leaning' in the same direction. The program was amended so that if new initiation points arose within a certain distance of an existing line the point was to be shifted onto this line. The pattern produced using this rule looked very similar to that seen on the actual shell: some examples of the computer patterns are shown in figure 2.8d.

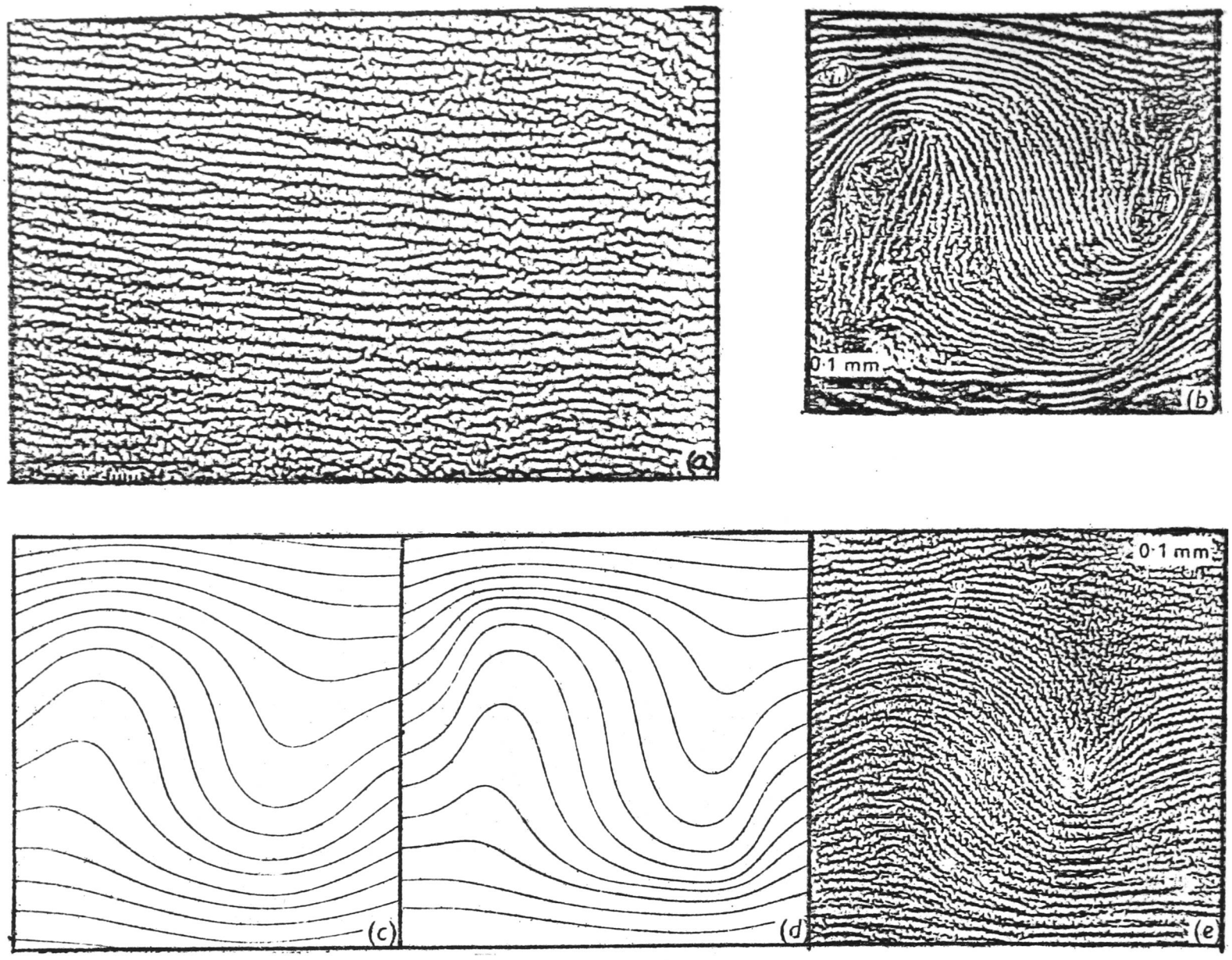
Waddington and Cowe point out that this pattern reproduction indicates only the logical structure and not the causal mechanism behind the production of the shell pattern. Their work has been carried further by Herman and Liu (1972) who have tried a severe test of the model - does it work for other, related but different-looking shell patterns? Herman and Liu used a version of the Lindenmayer model described on page 32 , with the

Lindenmayer filament representing the 'growing' edge of the shell (the right-hand most column of Waddington & Cowe's simulation). The successive rows of the pattern represent the states at the edge of the pattern at a given time, and by changing a "simple input parameter" (no details given) two other shell patterns are produced. One of the patterns shows large triangular dark areas, where pigment was deposited over a wide area for a period. The glat base of the triangles means that cessation of pigment formation is very sudden - Herman and Liu draw an analogy between this situation and the 'firing squad synchronization' problem in automata theory.

> "Program a finite state automaton in such a way that each element of a finite one-dimensional array of such automata will enter a specific state simultaneously, irrespective of the length of the array."

Herman and Liu have also simulated Wolpert's French flag analogy by using Lindenmayer models. This is done using reflecting waves of three different types. When one of the end 'cells' of a one dimensional array is disturbed, it sends out waves i, j, and k with speeds 1/1, 1/2, and 1/3 respectively. When the i wave propagates to the opposite end and reflects back, it meets the j-wave and the k-wave and the boundaries between the various parts of the flag are set up. If the array is cut, a new set of i, j, and k waves are sent to build up a new French flag.

Lawrence and co-workers (described in Lawrence, 1971)



(*a*) Cuticle from adult abdominal tergite of *Rhodnius*, to show oriented ripples. Phase contrast, scale = 0·1 mm.

(*b*)–(*f*). Adult ripple patterns after 90 degree rotation of square pieces of integument in larvae. (*b*) shows pattern after operation in fifth-stage larva before feeding. (*e*) shows pattern after operation in fourth-stage larva before feeding. (*d*) and (*f*) are equilibrium patterns for gradient model with homeostat and (*c*) for comparison with (*d*), assumes a diffusion gradient set up between a source and a sink and represents a contour map after diffusion for a certain period of time following 90 degree rotation.

FIGURE 2.9

Rhodnius cuticle simulation (Lawrence, 1971a & b)

have directly simulated a system which exhibits gradient-like properties, by using the cuticle of the bug Rhodnius as a marker for the activity of the underlying secretory epidermal cells.

The polarity of the individual epidermal cells is expressed in the oriented cuticle secreted by the cells (Figure 2.9a). Locke (1959) showed that the laterally oriented ripples seemed to be controlled by a gradient. If a square piece of epidermis from a larva in the final larval stage is rotated through 90°, the epidermis secretes an S-shaped curve of ripples in the adult cuticle (Figure 2.9b). The S or ∽ configuration of the S depended whether the rotation was clockwise or anticlockwise. Lawrence (1966) proposed that this result was caused by the presence of a concentration gradient of a 'morphogen' (Turing, 1952). If cells from a region of high concentration are placed near to cells containing a low concentration, diffusion occurs between them, and an intermediate concentration value is formed.

A computer model was devised to simulate such a concentration gradient, and to see if rotation through 90° would give a contour map like that seen in the Rhodnius cuticle. Several variations of the model were tried, to answer questions about how the gradient might be acting. Firstly, does the gradient depend only on the action of cells at the margins of the segment? The results of the model designed to test this showed that rather different S shaped patterns from those seen in the cuticle were formed (Figure 2.9c). The model also predicted that the greater the period between rotation and cuticle secretion, the more normal should be the cuticle (i.e. the diffusion gradient would slowly work its way back to normal).

This was not found to be so in the experimental situation. It was decided that some equilibrium must be maintaining the gradient landscape. A new model was, therefore, devised in which a 'homeostatic mechanism' in the cells tried to maintain the concentration of morphogen to the level it was at before the operation. The final equilibrium reached then depended on the success of this resistance: if the resistance is high, the equilibrium pattern will be S-shaped: if it is low, the pattern will not be so abnormal. (Figure 2.9d & f).

The simulation fitted well with this theory.

Lawrence's group then further suggested that the cells try to maintain a 'level' set at a specific stage of the cell cycle, and that the cell maintains this level until it again moves through the cell cycle. If, during this time a new level is set, the acquired new level in the cell will then be different from the old. As with Waddington's model, Lawrence shows that a logical mechanism can give a structure very like that seen in the living situation. This is one of the cases which, I have argued in the first chapter (p. 15) may be few in number, in which Wolpert's 'positional information' concept (Wolpert, 1969) readily applies.

Gradients are also the central factor in a model due to Wolpert (Wolpert, Clarke and Hornbruch, 1972). For many years, the results of the experiments on grafting and regeneration in Hydra have been explained by a gradient mechanism, and, as with the Rhodnius cuticle, Wolpert has explained this in terms of 'positional information' (Wolpert, 1969). In the hydra, the positional information is supposedly set up by diffusion of a morphogen from the head end. The morphogen specifies a

positional value which the cells pick up.

The Hydra may be arbitrarily subdivided into nine regions H1234B56F, with H representing the head, B the budding region, and F the foot. If, say, 4B56F is removed, and another head is grafted onto the cut end, and the first head is removed to give 123/H at various times, when, and under what conditions, will a new head regenerate from the 1 region? It is shown that if the second head is grafted on 8 hrs. before the removal of the host head, new host head growth is inhibited, but if it is grafted on after removal, the host head reforms. If only a 12/H is used, however, grafting can be carried out up to removal of the host head, in order to inhibit host head reformation. To find out if these findings were compatible with the diffusion gradient theory, a computer simulation was used. The computer model used a source-sink mechanism to provide a linear gradient in morphogen concentration, and tested the outcome of grafting experiments with the theory. A line of 'cells' representing a hypothetical hydra 20 cells in length was used, with all 9 regions consisting of two cells apart from the 1 region which contained 4 cells (measurements of the actual length ratios of each section were used to determine these values). The Crank Nicolson procedure (Crank and Nicolson, 1947) was used for solving the diffusion equations. The concentration of morphogen at the head end was set at 100 arbitrary units (this is the 'source' end), and the foot end (sink) was held at 10 units. Each separate region was then represented by its own morphogen concentration level.

From the experiments it was possible to work out the

period of time needed for regions to change type. This was done by removing the head, and after varying times removing the 1 region and grafting a head onto the latter. If two sets of tentacles were subsequently formed from the aggregate, then the 1 region had been determined to form a head. At 18°C, the 50% success rate for two-head formation occurred at 13 hours. This figure was used in the model to provide data from which the diffusion constant could be calculated. The model 'hydra' was then tested to see how it would behave when 'subjected to' the grafting experiments described above. The results obtained from the simulation indicated that the diffusion theory indeed fitted the observed model data. The model is clearly quite simple, and as Wolpert suggests 'should be taken as no more than making diffusion a plausible mechanism'.

A final model of pattern formation simulates the arrangement of wool follicles in the skin of sheep. (Claxton and Sholl, 1973). Again, a diffusion model is invoked to explain the origin of the primary follicle patterns, and the simulation shows patterns similar to those seen in vivo. Models have also been produced to investigate the expected sizes of patches in mammalian chimaeras (e.g. West, 1974 and see chapter four). Poodry, Postlethwaite and Schneiderman (1971) have employed a similar technique to investigate patch sizes in reconstituted fragments of imaginal discs.

4. SOME GENERAL NOTES ON THE COMPUTER MODELS DESCRIBED ABOVE.

Several points are brought out in the simulations, and

these might be summarised as follows.

(i) The computer enables us to make predictions about the outcome of hypotheses which cannot be adequately followed through mentally because they involve too many complex steps.

(ii) The simulation of fully deterministic processes is possible, as well as processes which, because of lack of detailed information, are best simulated by stochastic means.

(iii) There are often several physical manifestations of the same formal model, for example the similarity of Ede and Mitolo's models. Also, once you have the logical basis of a model to describe a system, it need not mean that the physical mode of action of the real system has been found.

(iv) One need not simulate all aspects of a system in order to produce a helpful working model.

(v) The various models show the ability of the reiterative application of 'rules' to generate complex forms. This may be a method of great importance in the study of development.

CHAPTER THREE

COMPUTER METHODOLOGIES

The sorts of simulation to be discussed here rely primarily on the spatial arrangement of cells - input and output of information to and from simulated 'neighbouring cells'. In this chapter, I will list and describe the various techniques I have used in making two-dimensional computer models of developing systems: a more detailed discussion of how such tools can be effectively put together and employed in specific situations follows in the later chapters. Rather than describe the basic features of cell interaction simulations individually, we will begin by looking at a simple model which uses some of the techniques employed in more advanced systems.

Growth in a one-dimensional filament.

The initial basic unit of all our simulations in this chapter will be the cell. It will be represented either by an array element, or group of such elements, in the computer. To represent a filament, such as an algal strand, in computer terms it is necessary to start by considering a one dimensional integer array. This consists of a variable number of elements that can be referenced in the form

List (1)..... ..List(N-1).. List(N),

where N is the number of elements in the array. Figure 3.1 shows how these elements might actually appear if the storage locations in the computer that they represent were actually drawn as a series of 'pigeon holes'.

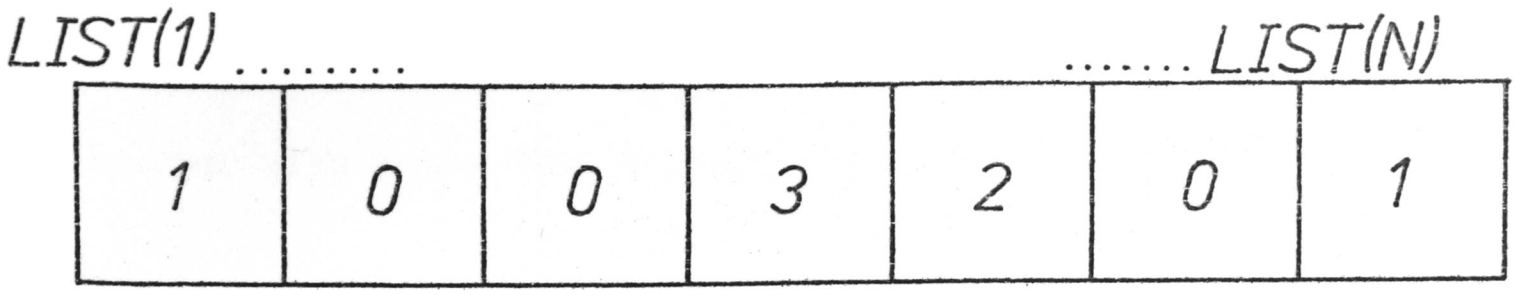


FIGURE 3.1

Each location can hold an integer number with a range depending on the computer to be used. We can make each location that we want to represent a 'cell' hold a special number (say, 1), and each location representing 'medium' or a 'space' contain a different number, say, 0. Figure 3.2 shows a short, idealized 'algal filament' represented in these terms.

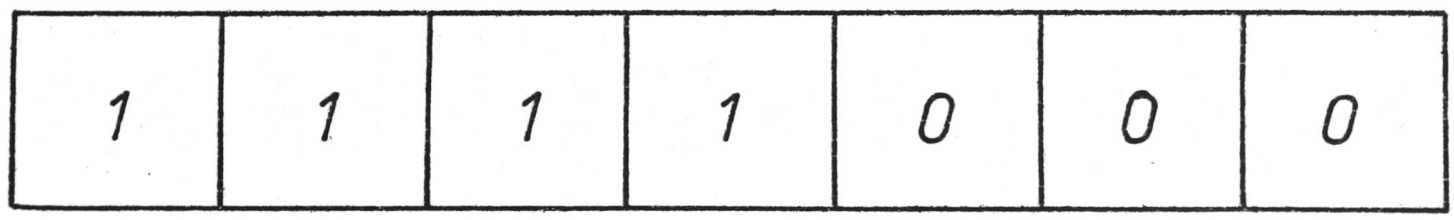
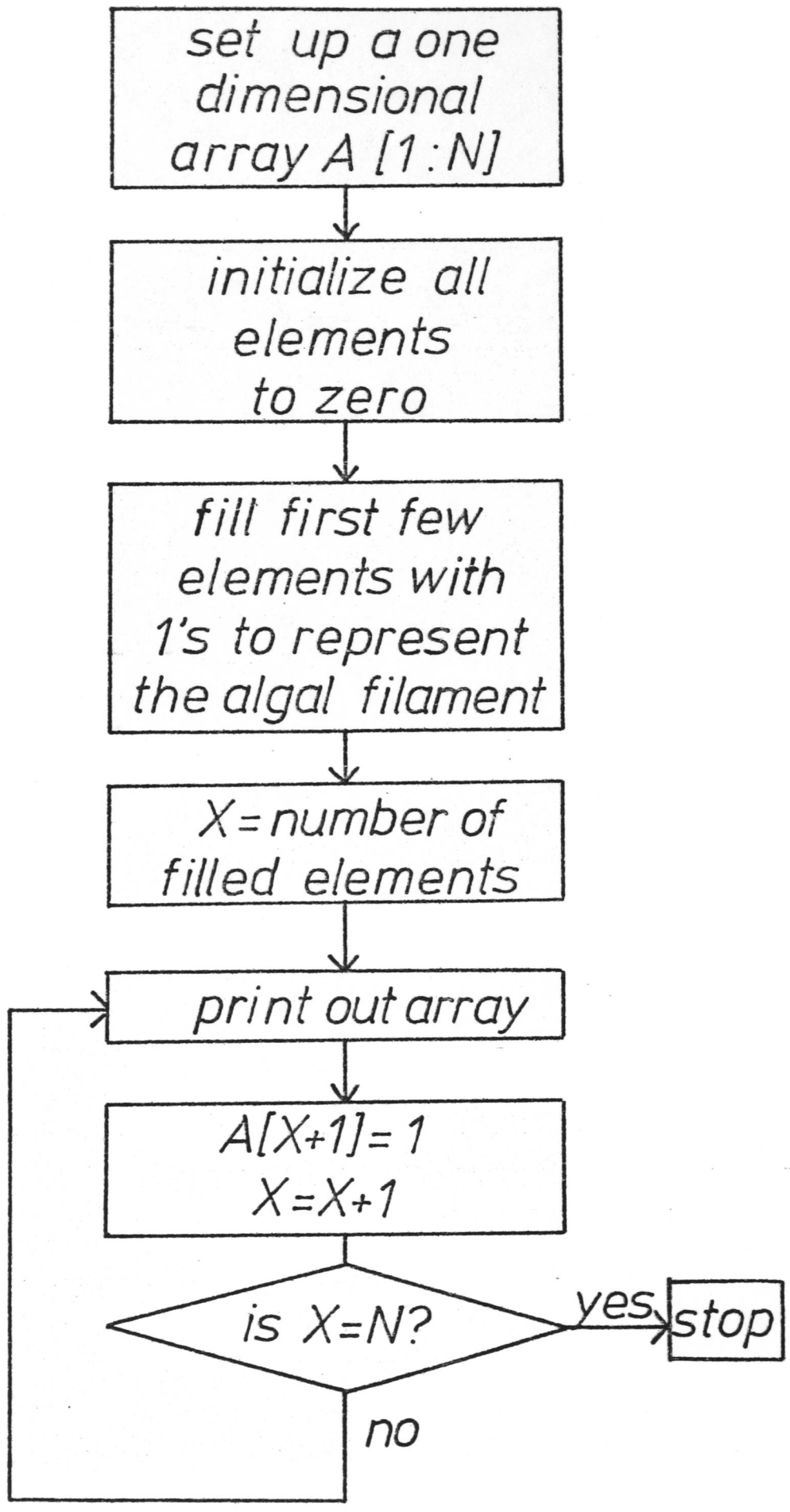


FIGURE 3.2


set up a one dimensional array A [1 : N]
initialize all elements to zero
fill first few elements with 1's to represent the algal filament
X = number of filled elements
print out array
A[X+1] = 1
X = X+1
is X=N?
yes
stop
no

PROGRAM 3.1

To 'grow' this filament, it is only necessary to replace each successive 0 by a 1. The flow sheet to do this is shown, together with a specimen printout (Program 3.1 and figure 3.3).

```
1 1 1 0 0 0 0 0 0 0
1 1 1 1 0 0 0 0 0 0
1 1 1 1 1 0 0 0 0 0
1 1 1 1 1 1 0 0 0 0
1 1 1 1 1 1 1 0 0 0
1 1 1 1 1 1 1 1 0 0
1 1 1 1 1 1 1 1 1 0
1 1 1 1 1 1 1 1 1 1
```

FIGURE 3.3

With such a simple rule no allowance is made for growth in intermediate cells in the chain - that is, to 'point' at a particular cell and tell it to 'divide'. This sort of sophistication only becomes necessary when it is desired to simulate a cell mass growing into some particular shape, or if more than one type of cell is present.

In fact, quite elaborate rules have been devised which grow '2-state' cells - cells which can hold one of two different numerical values representing different types of cells, depending on 'input conditions' - information received from the surrounding cells - into filaments. Systems of this type can

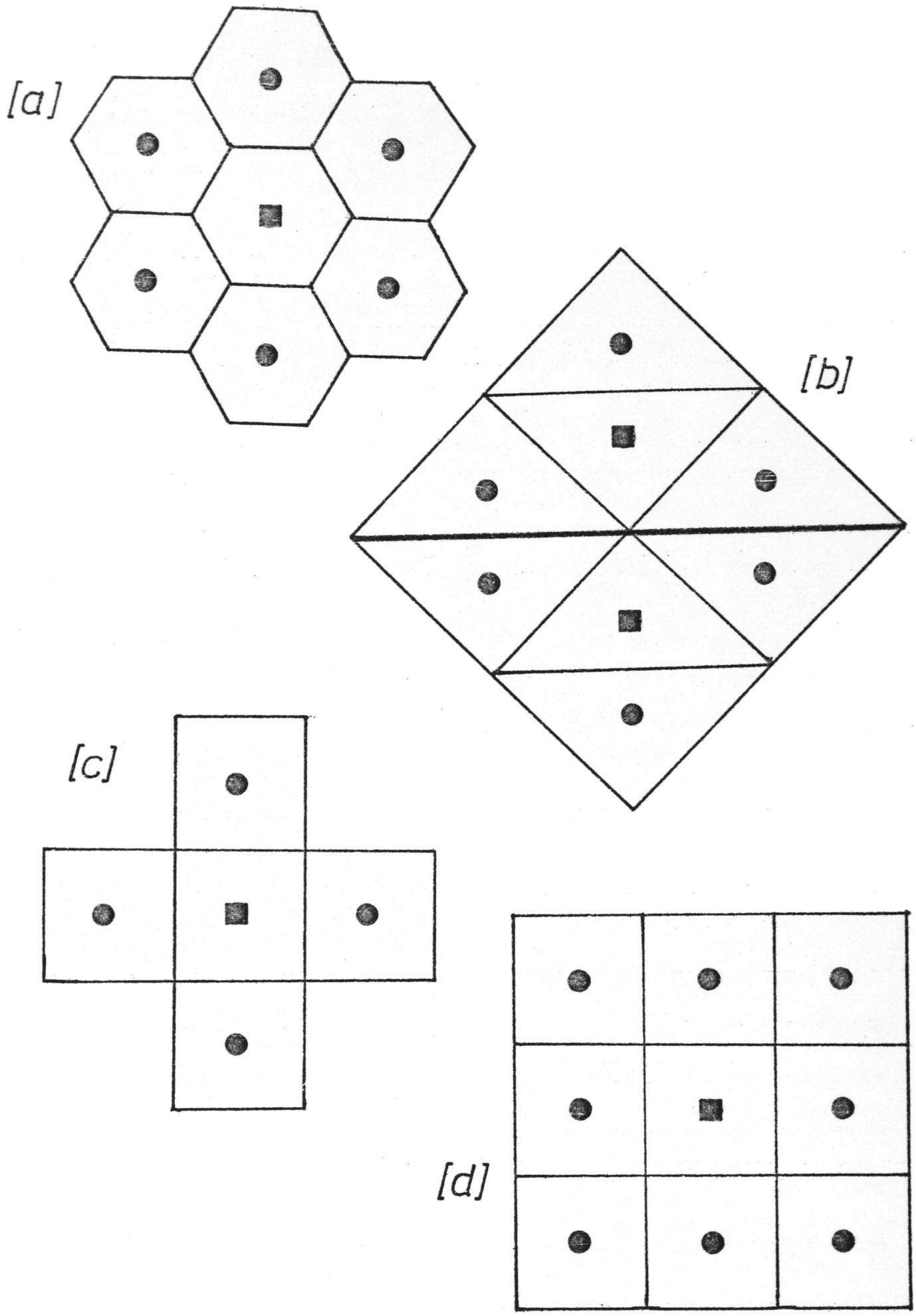


FIGURE 3.4
Types of cell packing in two dimensions: [a] hexagonal; [b] triangular; [c] square; [d] eight neighbour. The nearest neighbours of cells ■ are marked ●

produce complex patterns of 'differentiation' along the length of the filament, as shown by Aristid Lindenmayer, whose work we considered in the last chapter.

How many dimensions?

Having looked at the basic way of representing cells on a computer, let us pass on to a central question. Computers can deal with one, two or three dimensional arrays. Clearly, however, the complexity of programs will increase with the number of neighbours cells have to deal with. In the filament system, each cell has only two nearest neighbours, one to the left and the other to the right. In a three dimensional model, this figure can rise as high as 27. The implicit complexity involved in writing three dimensional programs means that most cell simulations have been carried out in two dimensions only. We have already seen in the first chapter that this need have no effect on the validity of a model of a three dimensional living system, so long as the experimenter is not trying to produce a 'mirror image duplicate' model of the real system on the computer.

Number of neighbours to each cell.

The number of nearest neighbours that each cell may possess in a rigid array is limited. In a two dimensional lattice, there are several configurations which may be used. It is normally taken (for example by Williams and Bjerknes, 1972; Antonelli et al., 1973) that cells tend on average to pack into a hexagonal alignment *in vivo*. Figure 3.4a shows that each unit of a two dimensional hexagonal array has six nearest neighbours.

There are two other regular tessellations of a two dimensional array: by triangles (fig. 3.4b) and by squares (fig. 3.4c). An eight neighbour space can also be defined (fig. 3.4d) - diagonal elements are also taken to be nearest neighbours. This produces an irregular tessellation, with the centre of the diagonal elements further from the centre of the central element in the ratio 1: 2. However, this will balance out for all elements in the pattern, and some authors (e.g., Leith and Goel, 1971) have used this arrangement.

Two dimensions out of one.

Two dimensional arrays are a feature of most computer languages and language implementations where large machines are in use. Computers usually process two-dimensional data more slowly than the equivalent data in a one-dimensional format and many small machines do not have the facility for handling data in this form. To make two-dimensional techniques more accessible, and to speed up the execution of programs, it is quite possible to treat one-dimensional arrays, or 'lists' as if they are two-dimensional. This is done simply by 'chopping' a long one-dimensional list into a number of segments, each segment corresponding to a successive row of the two-dimensional array. The number of each row is, therefore, the next increment on the 'y' axis, and the length of each row the maximum 'x' figure. This is illustrated in the figure below. (Figure 3.5).

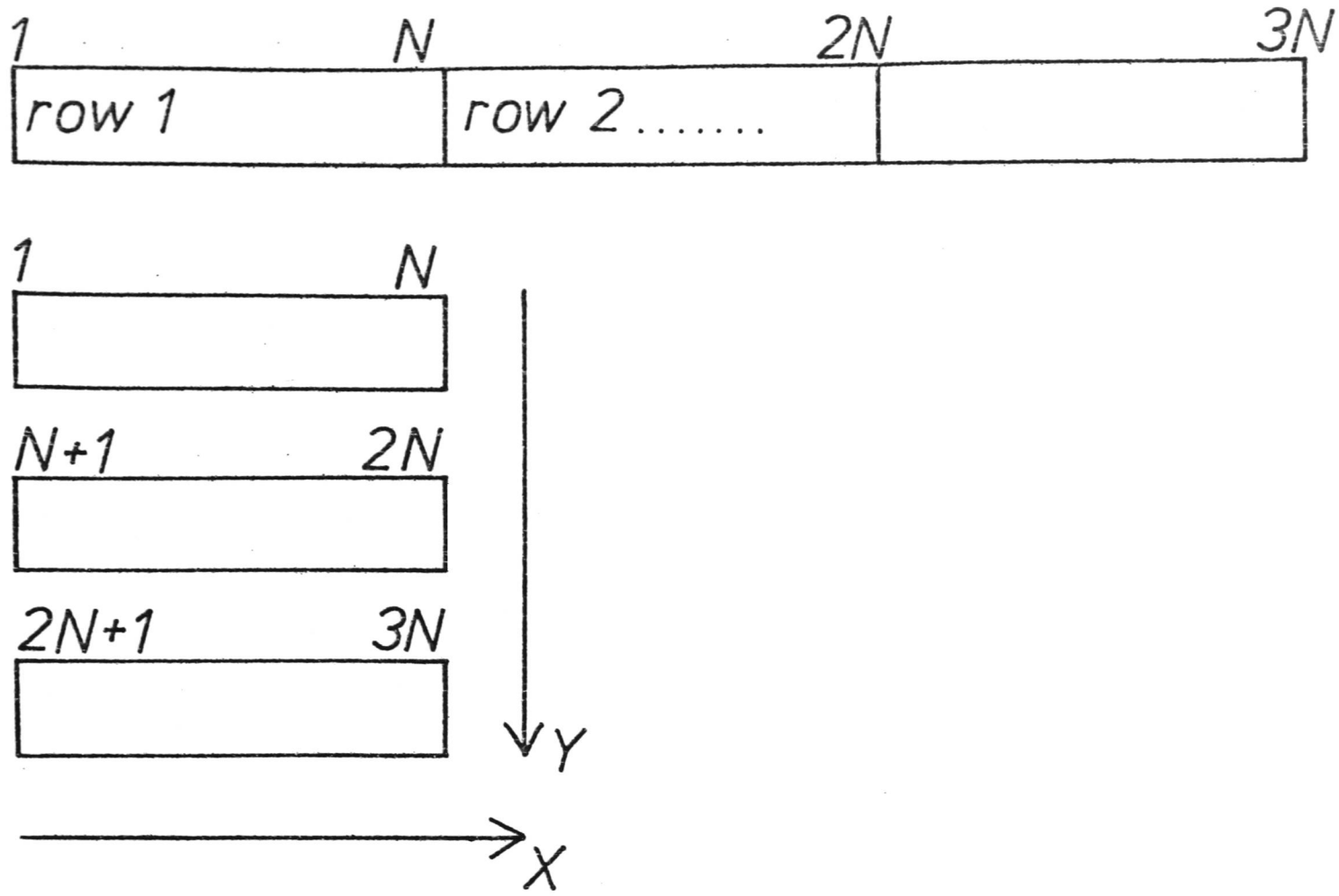
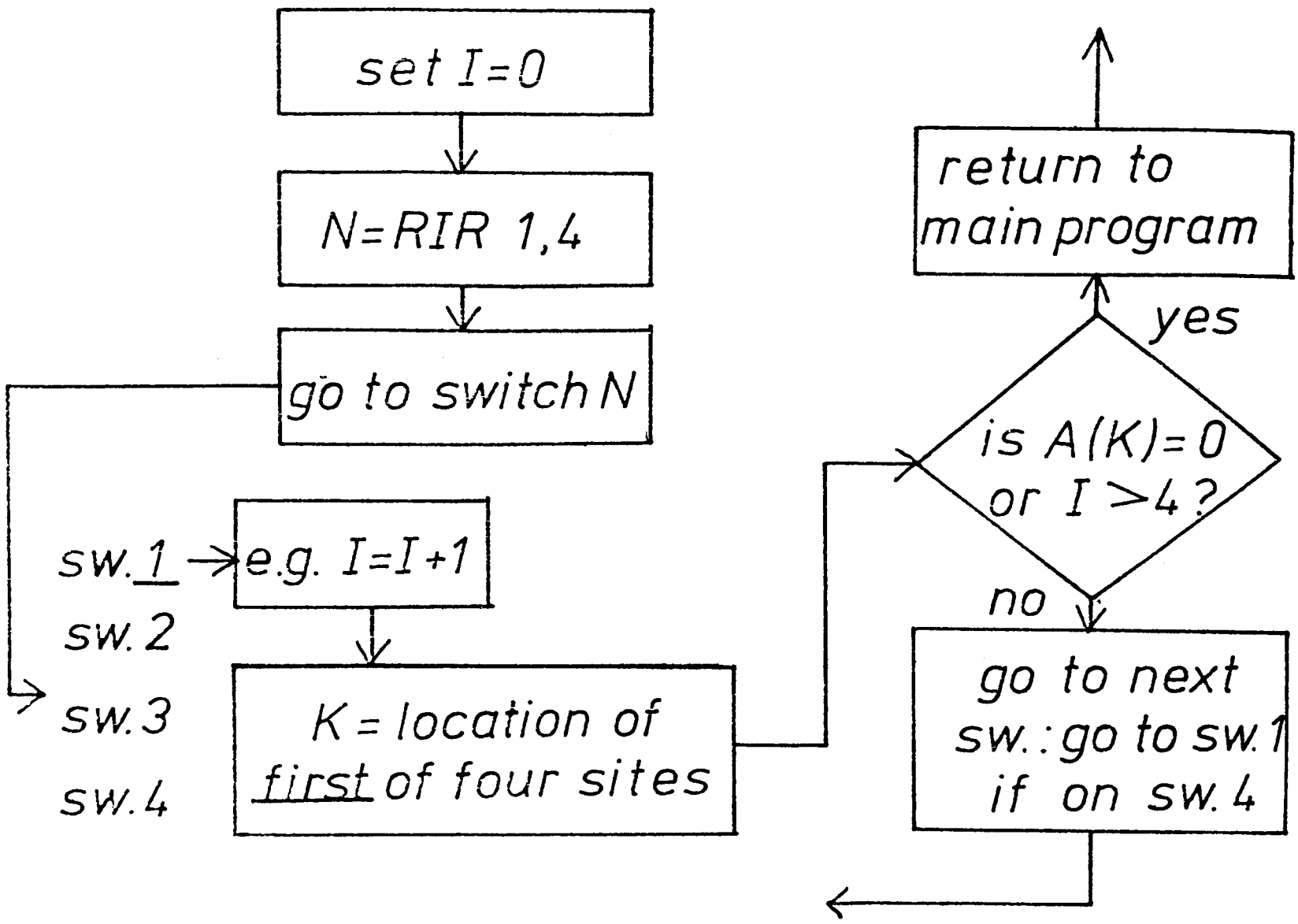


FIGURE 3.5

A simple two-dimensional cell population growth model.

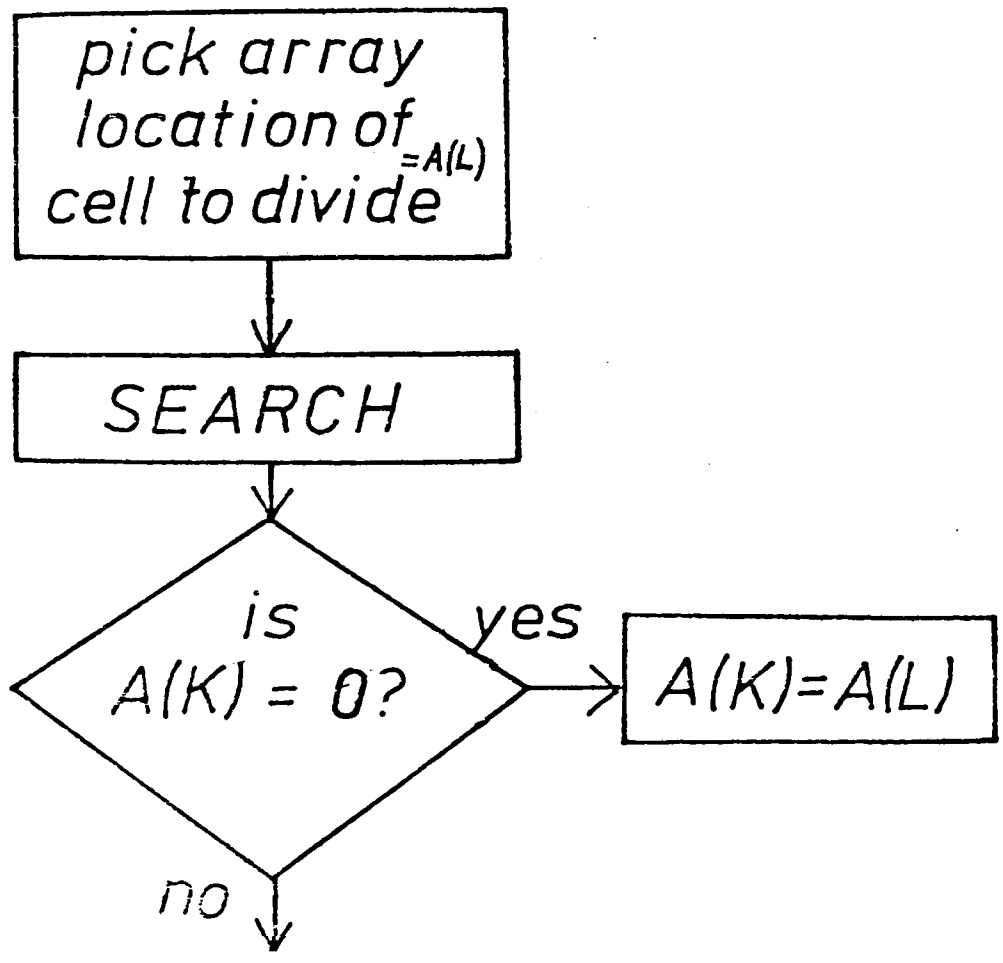
The first models devised to simulate cell population growth and morphogenesis were elementary in structure, and can be used to illustrate the one-dimensional 'chopping' technique and the use of array elements in a 'square' format. The initial step is to choose a suitable array size, say 40 x 40 integer units. In ALGOL a suitable one-dimensional array might be defined as M(1:1600). The array is then initialized with all elements containing the 'medium' or 'empty' value of 0. Next a 'seed cell' is placed in the centre of the rectangle of the array (this will be at about position 820. M(820) will now equal 1. The next requirement is a set of growth rules to enable this

Routine SEARCH



.and the main program link

PROGRAM 3.2



solitary cell to divide.

As this is a 'square' growth model, the cell can divide in any one of four directions. Obviously, when only one cell is present, there is only one choice of the next cell to divide. The cell to replicate in later stages is chosen by using a 'random picking' method: this technique will be described below.

Division takes place by making one of the neighbouring array sites equal in value to the 'dividing' cell. Hence the original site becomes one of the two 'daughter' cells. An assignment statement such as A(K) = A(L), where I is the array co-ordinate of the dividing cell, and I+1 the co-ordinate of the space this cell is dividing into, is used to simulate the replication process.

In this elementary model, we will assume that only cells with at least one free space in the immediate neighbourhood can divide, thus avoiding any worry about having to 'push' neighbouring cells out of the way to create space for a new cell to move into. The following example (Program 3.2) shows how a section of program can search for a space around a chosen cell, and allow the cell to divide randomly into one of the available spaces. RIR(X,Y) is the name of a function returning an integer random number in the range X,Y. A program written on these lines will multiply from the initial cell until the array bounds are exceeded (and the program is faulted). To stop faulting, we must test to make sure that each array position named in the routine exists. A good way of doing this is to fill the elements

in a single element width 'border' around the array with a dummy value (say, -1). This can be tested for before each division, and the site ignored if the array bounds are reached by trying to divide in a particular direction. In the model being described, a situation like this might be handled by the programmer telling the simulation to stop at the point the border is reached.

Other ways of handling edges exist. It is possible to fold the edges of a two-dimensional array onto a torus: this means that the effects of reaching a finite barrier do not exist. In some computer installations it may be possible simply to fault trap for illegal array bounds. The 'border' may even have to be more than one cell diameter in width if long-range cell interactions are allowed to take place in a model, otherwise cells may try to 'interact' with non-existent array positions. Unless all cells in a model system always have equal potential of dividing, there has to be some ordering process which controls which cells are able to divide at any time. Thus, we are introduced to the concept of a 'control list' governing which cells can divide and have divided in a 'generation'; this, in turn, leads to the need for a suitable time scale for deciding how soon cells can divide a second time after a round of replication. The most convenient time scale is the generation, defined as the period in which all cells have been allowed to divide once. The control list is a convenient method by which the division state of all the cells in the model can be determined. In the model at present being considered, the list must also include facilities for removing cells from the list when they can no longer divide (that is,

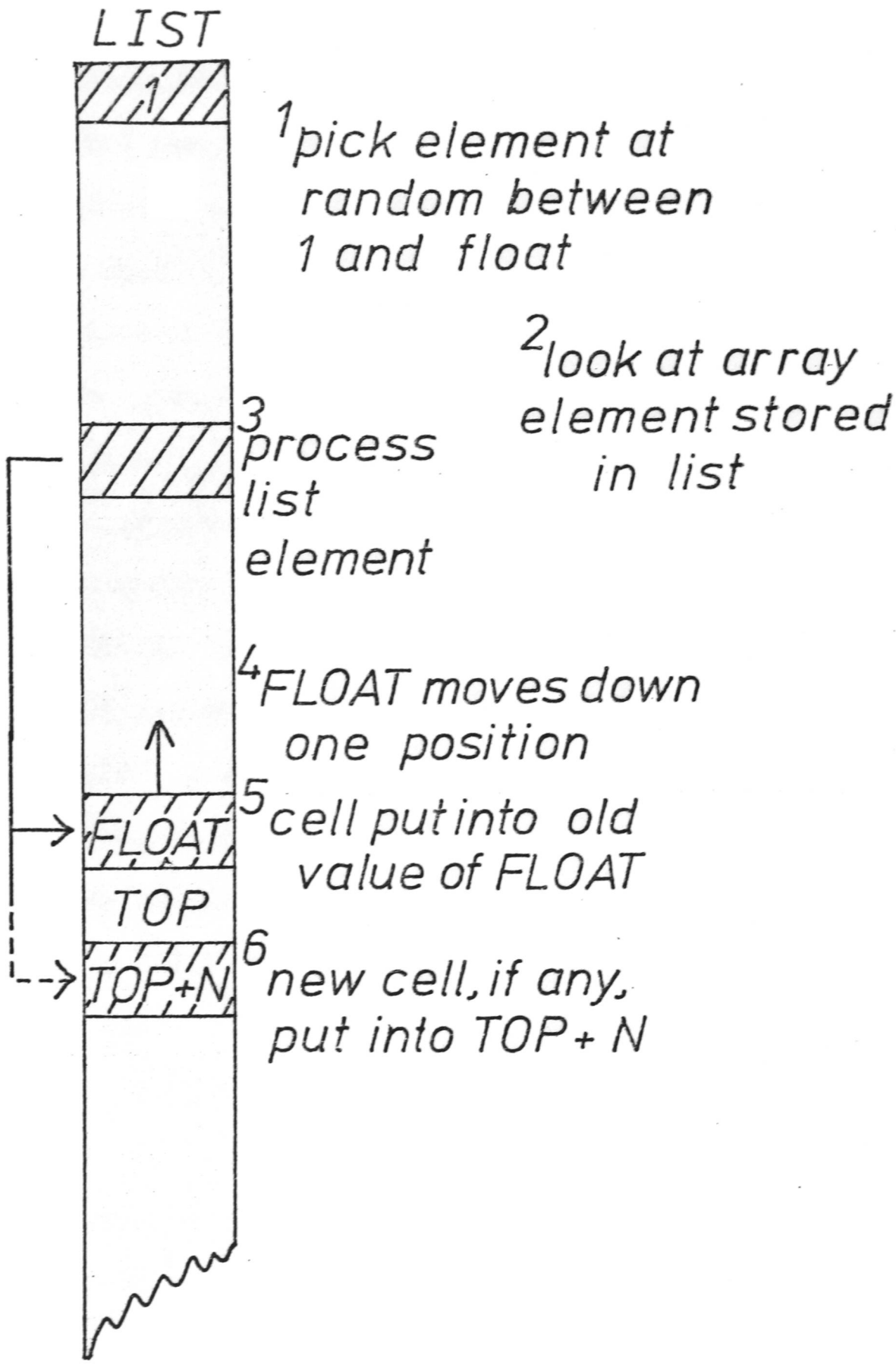

LIST
1
1 pick element at random between 1 and float
2 look at array element stored in list
3 process list element
4 FLOAT moves down one position
5 cell put into old value of FLOAT
6 new cell, if any, put into TOP + N
FLOAT
TOP
TOP+N

FIGURE 3.6

when they have become surrounded by other cells). In models where all cells can divide, the list must be used for all the cells.

A one-dimensional array is maintained, with each element storing the location of one of the 'cells' on the main array. At the start of any generation, the list will need to be twice as long as its length at the end of the previous generation.

The list is set up as follows (fig. 3.6). At each new generation, the number of 'cells' present becomes equal to a variable we will call TOP. The list locations 1 to TOP then become filled by the locations of the array points on the main array that contain cells. A second variable FLOAT is set to the same value as TOP.

All the list elements between 1 and FLOAT are then processed in the following manner. A number between 1 and FLOAT is chosen at random (this is the random step by which the cell to divide was chosen in Program 3.2). The cell in the main array location stored in the list element chosen then attempts to divide (Program 3.2). If this is satisfactorily done, then all the list elements between the one picked and FLOAT inclusively move down the list one position, so removing the chosen element. FLOAT is then decreased by one, and the processed element is placed in the old FLOAT position.

If the cell has divided, the daughter cell has its lattice co-ordinates stored in list location TOP + N, where N-1 is the number of daughter cells that have already appeared in the present generation. If no division is possible, for some reason, the same procedure occurs but TOP + N remains unused.

Although this procedure has advantages in 'neatness' it should only be used when speed of operation of the program is not essential. This is because, for every division, an average of $\frac{1}{2}$ of the cells which were present at the start of the generation will have to be moved down the list one position. A quicker way of carrying out the 'cleaning up' procedure, by which dividing cells are placed at the bottom of the list, would be to let, say, 10 cells divide. These cells are then processed in the same way as before except that their original positions are marked by some dummy variable, for example '9999'. After the given number of divisions, the whole array is swept through once, and all '9999' positions are excised. In this way, about $^{9}/_{10}$ of the laborious array position changing can be avoided. If a '9999' is hit during random casting for cells to divide, a new cell is picked to divide. The printout of the simulation after one, three and six 'generations' is shown below. (Fig. 3.7).

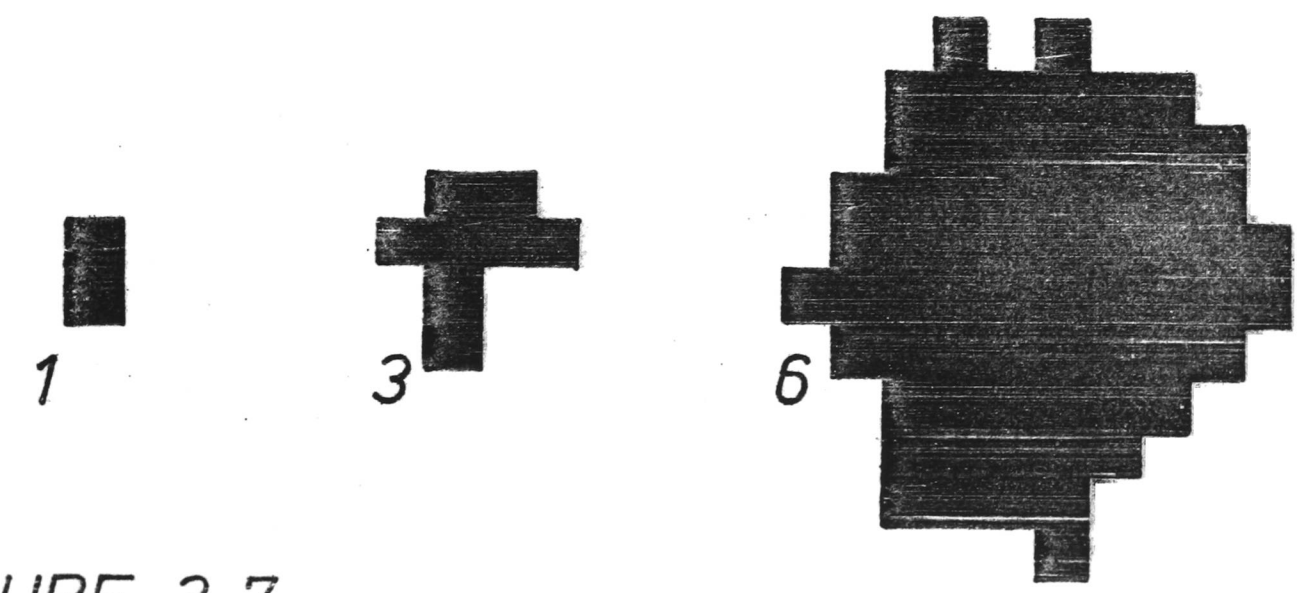
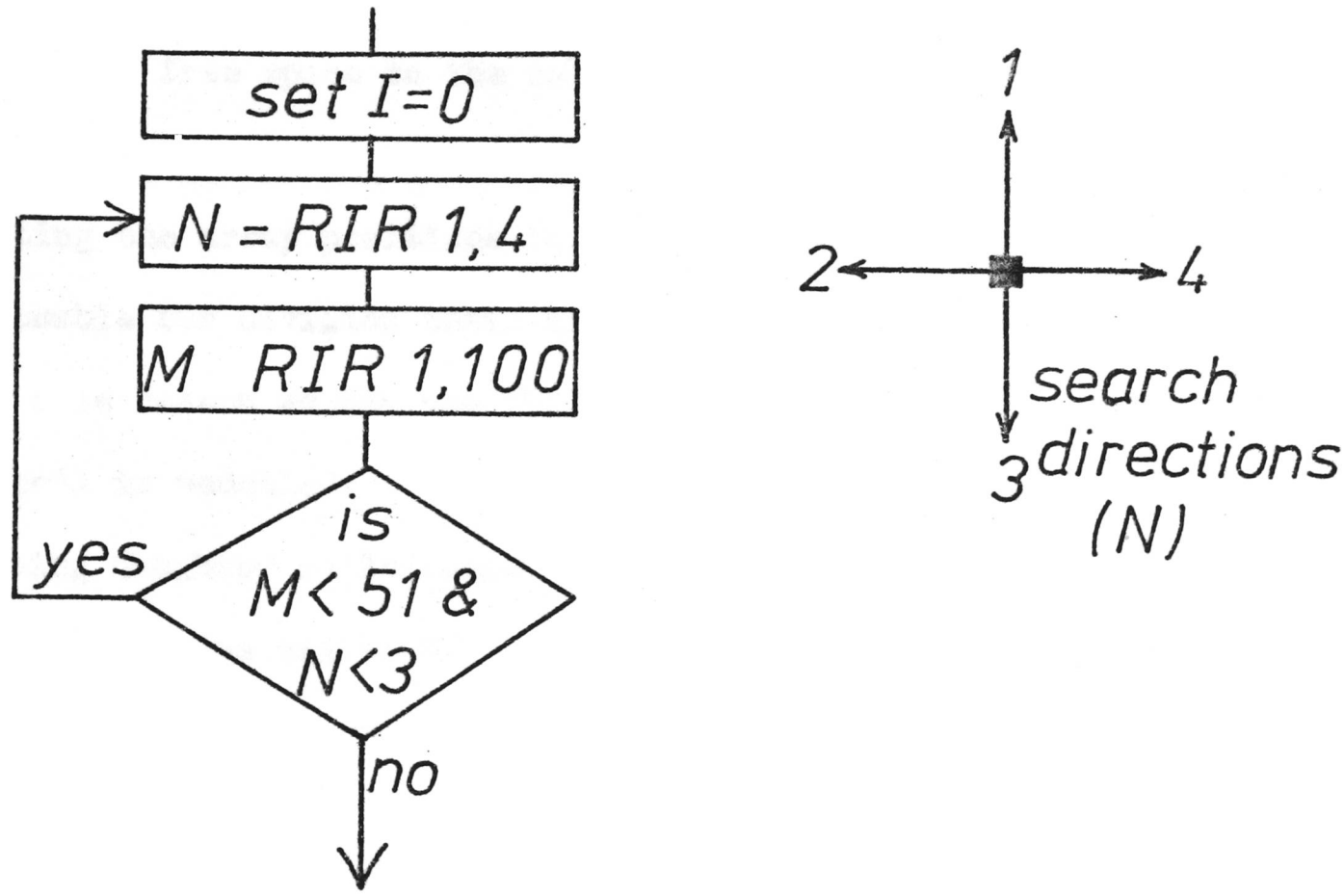


FIGURE 3.7

It is only in variations on this simple scheme that the first uses of such computer models become clear. Suppose we ask certain questions like - what happens if the computer cells divide preferentially in certain directions, or only divide if they are surrounded by less than a certain number of cells in the immediate neighbourhood? Preferential division is easily programmed into the model just described. All that is needed is to include a probability of preferential searching in one or more directions. The start of program 3.2 might then read (Program 3.3).



PROGRAM 3.3

This would give a 50% greater probability that searching will take place to the right, and below the parent cell.

A model that allows all cells to divide.

The main limitation of models of the type described above is that only cells with free edges can divide. If only one cell type is to be modelled, an alternative procedure can be used. This has the facility of allowing cells inside a cell mass to divide (after a fashion), without recourse to more complex 'pushing algorithms' needed to clear a space for the daughter next to the parent cell.

Cells are picked from the control list in the same way as in program 3.2, but instead of being dropped from the list when they become surrounded by other cells, the program finds the 'nearest' free space to the cell to divide and puts the daughter cell in this space. The free space is found by successively testing the array positions in each of the four directions allowable for division until it locates an empty space. Note that the sole reason we can use this technique is that only one type of cell is modelled: no way of simulating 'cell lineage', by placing daughter cells beside the parent is allowed for. The method is quite useful for less sophisticated models, and has been used to good effect by both Eden and Ede (see chapter two).

Differentiation on the computer.

Up until now, we have considered the array elements as carrying a 0 or 1 signifying whether or not they contain 'cells'. Each array element in the computer is a computer 'word' that can hold digits up to the word length of the computer being used, and so is capable of holding much more complex data than a single digit. In the following pages, both multi-array location cells

and 'bit packing' to get more information into one array element will be described. Prior to this, we will look at a program that uses two or more different 'types' of cells, each type being represented by a different numerical value.

Simulation of a cell aggregation system.

Several new factors are introduced in considering models of this type. 'Cells' must be able to recognise their neighbours in some way, they must be capable of movement in response to information perceived about the neighbouring cells, and they must have the ability to 'stick together' in specific situations. My own preference in modelling cell aggregation has been to use an eight-neighbour space: this is because each cell has access to sufficient neighbours to give an approximation to the cell-cell recognition process.

Cell aggregation has been studied by several different research groups, and in the previous chapter we looked at the different methodologies used; much insight into the basis of model abstraction can be gleaned from a careful study of these various approaches.

Let us look at the first difference between the present model and those described previously. Recognition has already been introduced in a disguised form, because the mere act of looking around a cell to see if it has a free space to divide into implies some kind of 'perception' by the cell hoping to divide. In the present example, we need to see how many neighbours of each cell type surround each cell, before deciding whether the cell is to move or remain stationary.

A simple example might be set up as follows: take a one-dimensional array of size N^2 elements and make $\frac{1}{2}$ this number of elements in the array equal to '1'. Set the rest equal to '16'. The rules for aggregation are then as follows. Each cell is processed sequentially at random (if required, a list similar to the control list for dividing cells can be used to make sure that all cells are processed once before any cell is processed a second time). 'Processing' consists of searching the neighbouring sites to see if the cell is in a clump, or stable configuration: this being preset by the number of neighbouring cells of the same type as the cell to divide necessary for the cell to be stable. A convenient way of doing this searching might consist of adding up the values of the surrounding eight array elements, putting the total in a variable called SUM and shifting SUM 3 bits to the right to find the number of '16's'. The number of '1's' can be obtained either by subtraction or by masking off all but bits 1 to 3 of 'SUM'.

The program can now check these two numbers against two preset constants holding the values determining how many cells of each type in the surrounding eight are necessary for the cell to be stable. If the cell is to move, a simple instruction will allow entry into a routine MOVE. MOVE may entail either random movement or some kind of weighted movement towards a higher concentration of cells of the same type as the cell to move; in this way, either random cell movement or directed movement can be simulated. Weighted movements can entail searching procedures over several cell diameters away from the cell to move.

It will be noticed that a model of this type introduces no fresh information onto the array. We will call this type of array bound simulation a static system. The converse, where division takes place and new cells are constantly being introduced would be a dynamic system.

Cell clone models - true dynamic systems.

The simple division systems described earlier are very limited in scope. We now come to discuss two more advanced types, both with their own pitfalls and advantages.

The one cell/array position pushing model.

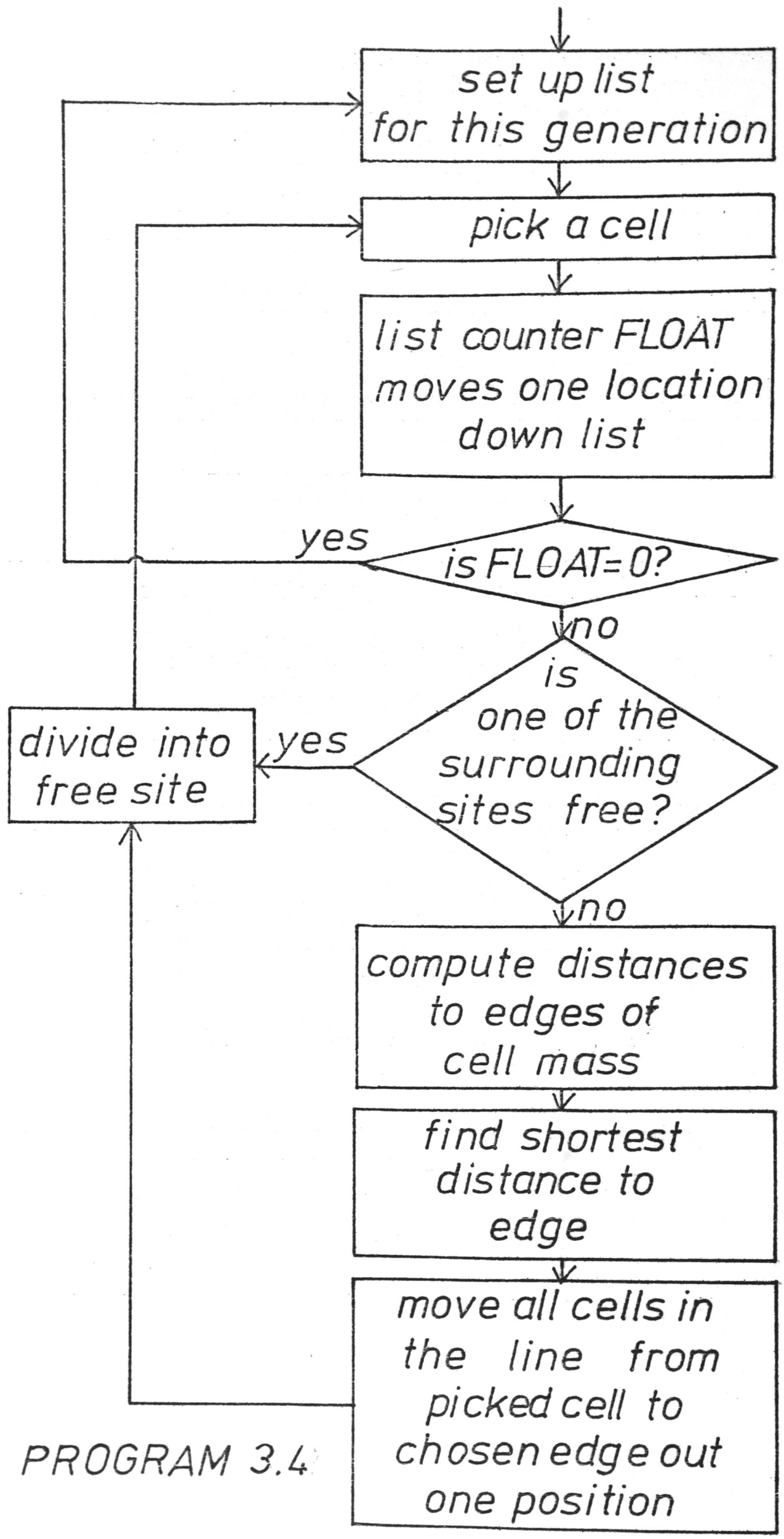
The disadvantages of placing daughter cells on the nearest available free 'edge' of the clone have already been mentioned. To overcome them, it is necessary to clear a space next to the cell to be divided. This requires thought, because all the cells in any system, be it a two-dimensional computer array or a living organism, have a definite spatial relation to one another. The computer array is a rigid lattice, the use of which lets us give our abstracted 'cells' some kind of spatial co-ordinates: However, the nature of the array makes it well night impossible to carry out division of the sort seen in living organisms (or even in monolayer cultures, the closest real-life equivalent to the two-dimensional simulations).

To illustrate this point, consider a mass of typically amorphous shaped embryonic cells. If one of the central cells in this mass divides, there will be an incremental change in shape of a large portion of the cell mass, as a space is allowed

for the new cell. If a computer 'cell' X 'divides' into a space Y, which is already occupied by another 'cell', and this latter cell is moved, it has to travel a whole array position in distance, which means the same thing has to happen to one of its own neighbours in turn, and so on out to the edge of the clone. There is, then, a marked difference in these two situations, and the modelling of cell divisions within a clone either has to allow for this 'single cell pushing single cell' phenomenon, or has to model cells as taking up more than one array position, so that 'flexibility' can be introduced. In this way, the sort of pushing produced by a single cell division giving rise to an incremental position change in a number of cells, as seen in living tissues, can be modelled. As direct cell/cell pushing is easier to program, we will look at this technique first.

A central question to ask is, of course, can the 'pushing' type of movement ever be analogized to any growing tissue? The answer to this is best illustrated by direct description of the use of a model employing pushing in a specific experimental context. Such a model system is described on page 114, and indicates that the effect of many pushings in a large simulated cell mass may not be very far removed from the incremental 'jostling' seen in living organisms.

There are two variations on the pushing theme. In the first, the cell computes the nearest free edge to itself, and all cells in the line between the free space and the cell to divide are moved outwards one position to create a new free space. The daughter cell can then be placed in this space. The most convenient way of programming the successive movements of the


set up list
for this generation
pick a cell
list counter FLOAT
moves one location
down list
is FLOAT=0?
yes
no
is
one of the
surrounding
sites free?
yes
divide into
free site
no
compute distances
to edges of
cell mass
find shortest
distance to
edge
move all cells in
the line from
picked cell to
chosen edge out
one position

PROGRAM 3.4

intervening cells is to move the outermost cell first - clearly this would be ludicrous in any real division system, but it leads to the same end product. (Figure 3.8 and Program 3.4).

An alternative procedure, although more costly in computer time, would be for each cell in turn to compute the nearest free edge, and push itself into the position occupied by its neighbouring cell in this direction. The neighbour, with no real 'position' would have to repeat this procedure. The advantage here is that the path to the edge from the cell that initially divided is rarely a straight line, thus 'slicing' by constantly pushing a regular file of cells is avoided. I must add here that no practical evidence exists that the latter method produces any noticeable difference in the type of clones produced, although its use is obligatory when using multiarraylocation cells (see below).

Using programs of this type, it is possible to 'tag' cells (by making them of a different numeric value), and to observe 'clone formation' by these tagged cells. The method can be used for any number of different clones, because the daughter cells always take the same values as their parents.

Constraints.

We have already discussed (page 70), how the provision of a 'border' can prevent cells dividing in particular directions. This effect can be modified, and used to model clone growth under physical constraints simulating, say, membranes or surrounding tissues; both of these have been shown to be important during embryogenesis. The simplest kind of 'physical constraint'

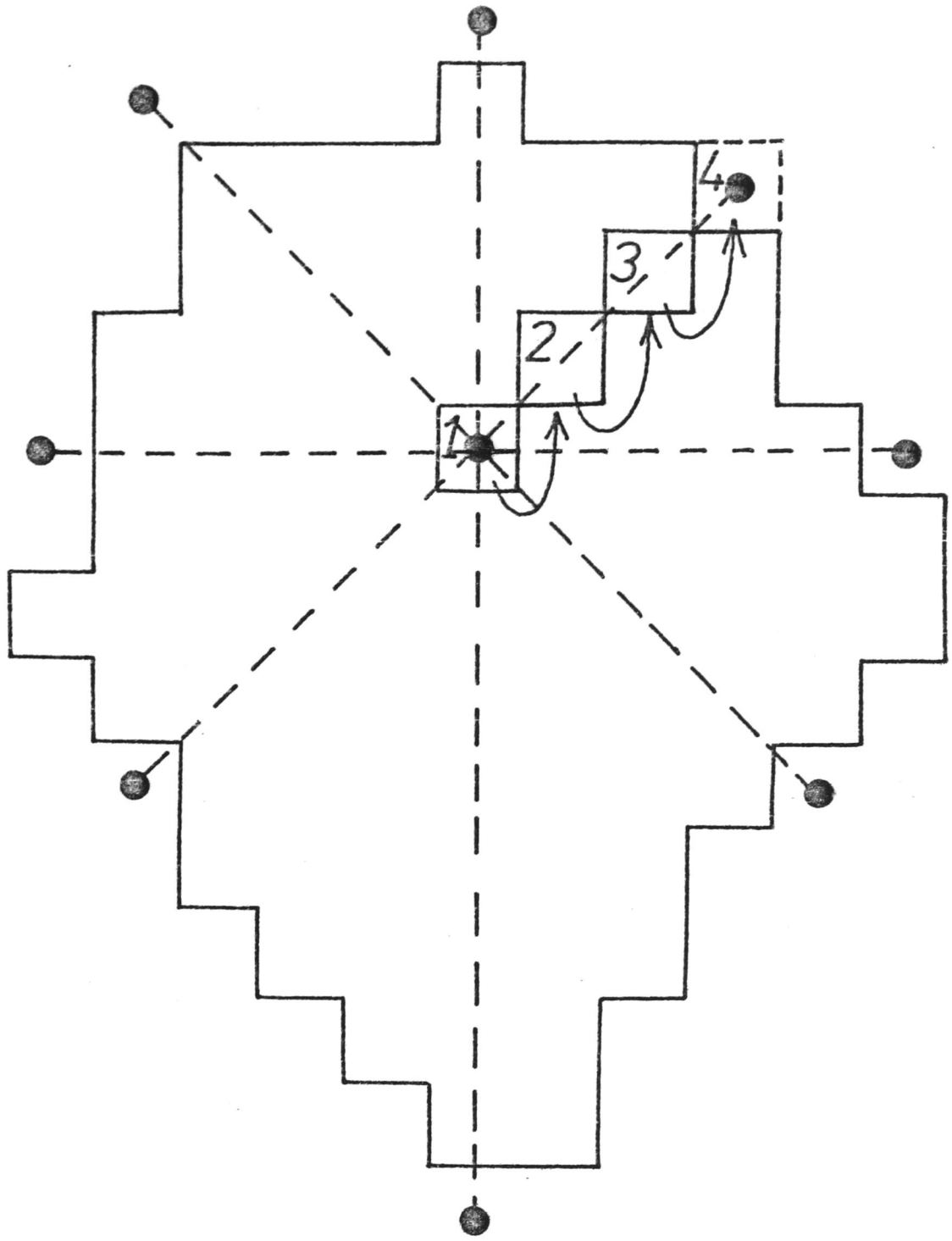


FIGURE 3.8 'Clearing algorithm'. 'Cell' to divide (1) finds nearest edge of colony by adding up numbers of cells in the 4, 6, or 8 possible division directions. (8 are shown in this example). The cell decides to divide top right. Cell (3) moves to space (4), cell (2) moves to space vacated by cell (3). Cell (1) can now divide into the space left by cell (2).

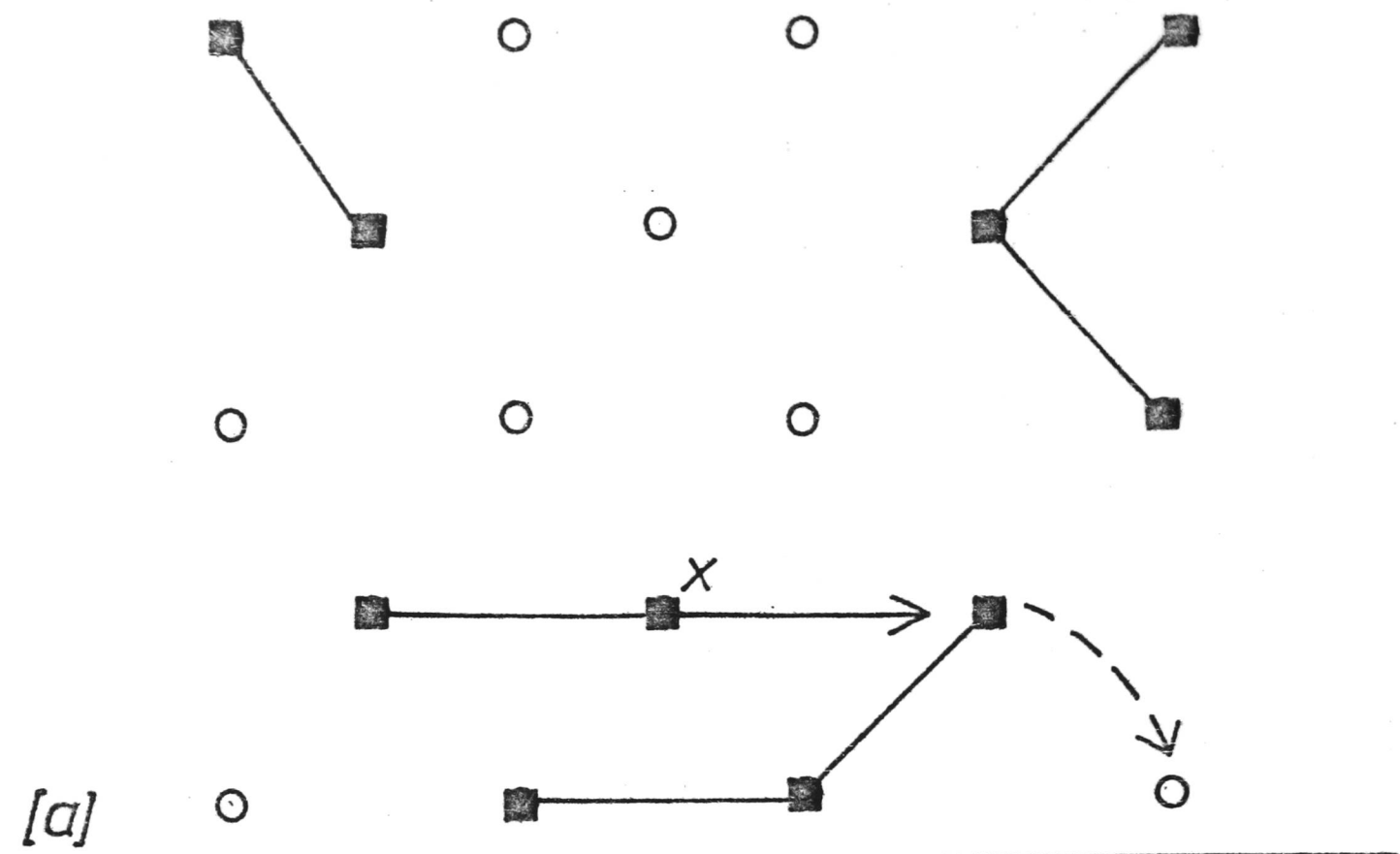
model is the simulation which prevents or limits cell growth in particular directions, and this is analogous, to a certain extent, to the external forces of membranes. It may be that a curved or 'growing' membrane is to be simulated, and this will not be easily reconciled to such a simple technique.

An ideal way of simulating a growing constraint is to embed a formula defining a geometric shape, like a circle or ellipse, into the program. It is then possible to increase the size of this constraint, and follow the growth of a cell mass 'deformed' by the external 'pressure'. An example of a program of this type will be discussed in chapter five.

Constraints are modelled by defining an area as the 'free' array (all locations in this area not occupied by cells at any time will have the value 0). The part of the array external to this area takes the dummy value -1 in its array locations, and all the array locations in this region are unavailable for cell division until their values are changed to 0.

'Complex' cells.

The disadvantage of one cell/array position movement by pushing is clear. It is possible to surmount this difficulty by using more complex cells and division procedures. Complex cells may, of course, be made up of any number of array positions greater than one; the easiest to describe and program contains a maximum of three array positions per cell. A model of this type can even include facility for cell 'growth' before replication - cells do not suddenly double in size, but gradually increase in volume before 'splitting', and the computer cells described up to



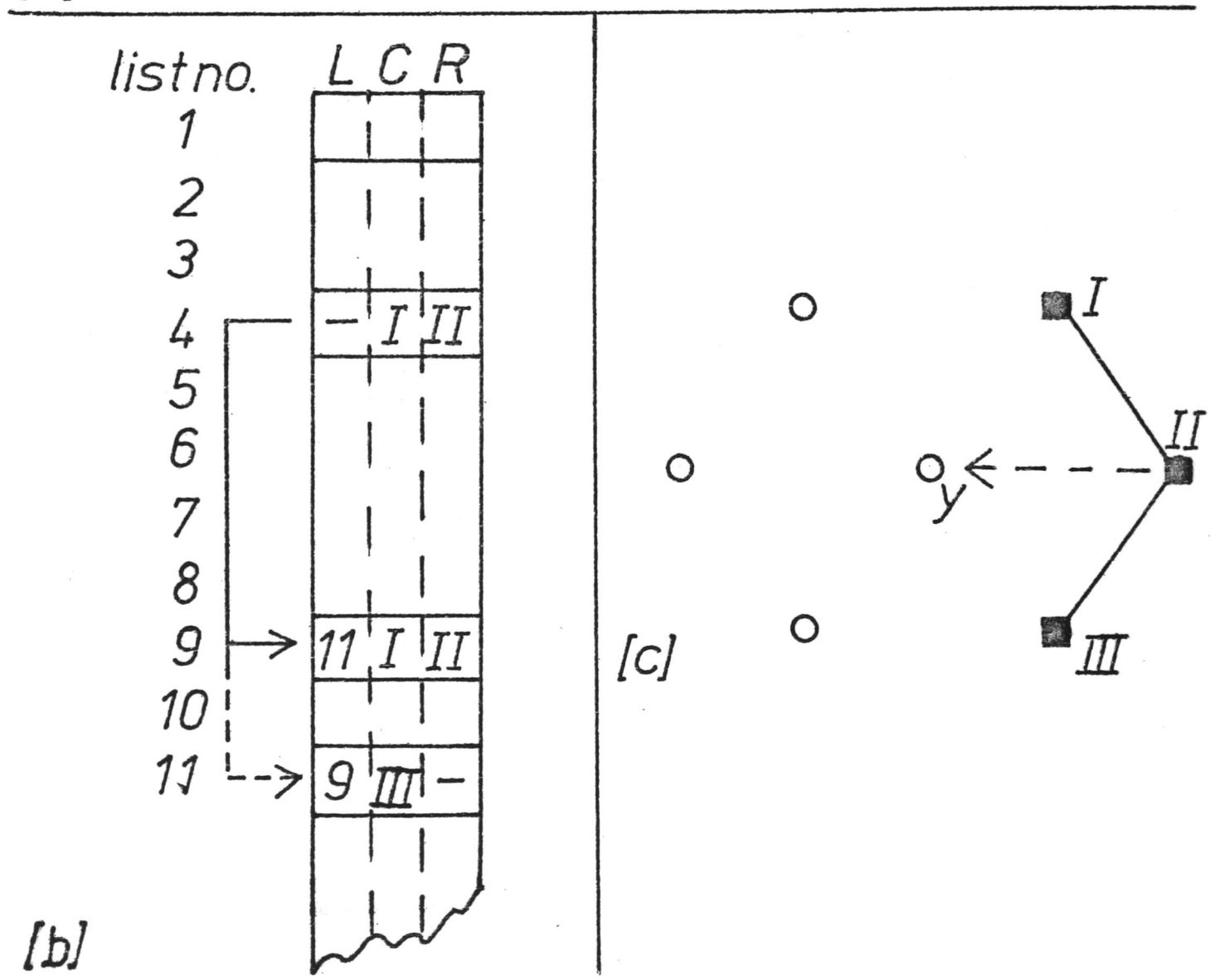


FIGURE **3.9** . Complex cells. (a) Both 2 and 3 unit cells are shown. When the cell marked X 'grows', the cell unit occupying the space chosen for the new unit must move. (b) List used for handling complex cells (see text for mode of operation - essentially the same as in figure 2.6, apart from word packing). (c) Relationship of units held in the shown list. 'Pivot' unit (II) can only move into site Y if required to move.

this point do not take this into account.

Picture a hexagonal lattice, with any two lattice positions 'joined', as in figure 3.9. Such a pair of array elements make up the basic 'cell'. The 'cell' can increase in size by one of the two halves of the cell 'duplicating'. A further duplication then leads to splitting into two 'basic cells'. It can be seen that a variety of 'shapes' can be produced by these cells. The most useful point is that the cells can now be 'pushed' without having to move their whole mass (figure 3.9a), so modelling the deformation property of actual cells.

It may be worthwhile here describing how the data referencing the complex cells is held in the computer - the techniques used may be of some use in similar situations. The cells in the model are initially made up of two array elements, as shown above, and these are held in the same format as we have seen previously - a main 'array' and a 'list'. The list is rather more involved in this case, however, each list element computer word being actually three separate pieces of information compressed into a single word. (See figure 3.9b). The right-hand section carries the main array co-ordinate of one of the component cells and the centre section holds the co-ordinates of its partner. The third (left-hand) section is initially left unfilled.

When the cell 'grows', the new part of the cell takes the fresh list element (TOP+N'), as in previous models. In this case, it 'occupies' the centre part of the word. This new element is placed in position TOP + N, as before, and its parent list element

takes the old FLOAT position. The left-hand parts of both words now take the value of the list position of the partner; the daughter takes that of its parent, and the parent location does vice versa. Now to carry out this 'growth', one of the two parent units has to divide in much the same way as in previous cases. The parent unit in question now becomes the PIVOT unit, and automatically moves to the right-hand end of its list location (swapping with the existing right-hand unit if it was in the centre, or staying in place if already at the right-hand end). This other Heath Robinson looking affair does have a motive, simply that if a cell divides or grows in the centre of the cell mass, it has to push itself a space. If all cells are one array unit only in size, there is no problem - cells in this type of model can move about freely. With complex cells, it may be that part of the cell may be physically separated from the rest of the cell if this rule is not modified, and so one of the units of the cell is designated a 'pivot'. The 'pivot' unit can be moved only to positions where it is still in contact with the other units of the cell. In figure 3.9 (c), for example, the pivot unit is only free to move to the site marked Y. The other units of the cell are free to be moved, so long as they remain in contact with the 'pivot' unit.

The pushing rules used with this model are modified from the 'each cell computes the nearest available free edge' technique. A word of caution is needed - this type of model is slow in operation, because of the many list element retrievals that have to be made - also, using the program to look at clone

growth gave no significant advantage over the less costly simple pushing model.

A word on 'continuous' cell modelling.

There are distinct advantages to using arrays for modelling cells - it is possible to spatially plot where cells are at particular times, simple rules may be recursively applied to see what patterns are produced, using known rules, and one can see the results of such simulations (see the section on 'input and output' of developmental models). The main bugbear is the huge jump between cells modelled by a single or few array positions and the living cell with its highly fluid structure. At present, therefore, we can only use two dimensional array simulations in a very abstract sense, and in the next chapter I will illustrate how the methods described in this chapter can be put to use.

CHAPTER FOUR

CELLS, CLONES AND PATCHES: AN ANALYSIS

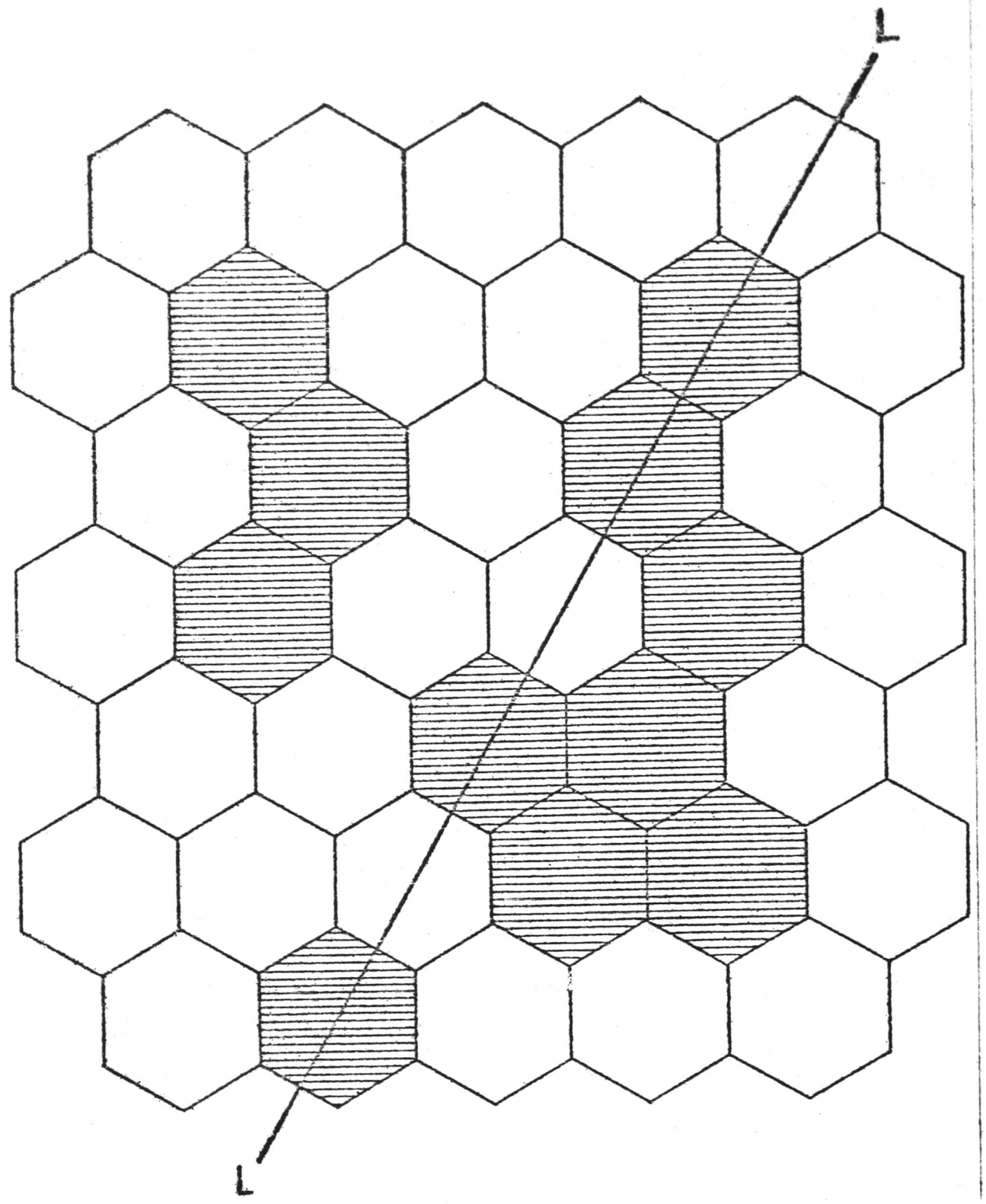
The following chapter is a 'preprint' of a paper to be submitted to the Journal of Theoretical Biology. It has been written with the co-authorship of Dr. W. Hill and Dr H. Kacser. Dr. Hill was mainly responsible for the statistical parts of the paper and Dr. Kacser for the overall form and structure the paper has taken. My own contribution was the design and execution of a number of computational techniques used both in this paper and in one by J. West (submitted to J. Theo. Biol.)

INTRODUCTION.

A number of experimental techniques are available which may throw light on the developmental history of cell lineages during the ontogeny of an organism. They involve the 'marking' of cells either by pigment or by enzyme identification. Thus, in Drosophila, somatic crossing-over of flies heterozygous for a gene lacking pigment forming ability has been used. By irradiation of such flies at various stages of development the frequency of crossing-over can be greatly increased and mosaic animals showing pigmented and unpigmented areas are produced (Becker, 1957). Similarly, the hypothesis of X-chromosome inactivation in mammals (Lyon, 1961) postulates that at an early stage of development, one of the X-chromosomes is functionally inactivated. If the two chromosomes differ in some of their phenotypic consequences (for example, pigment formation) the resulting animals are mosaics. Alternatively, experimental aggregation of two early embryos, genetically distinct by some identifiable product, can produce chimaeras which again show patches of two types of cells. (Tarkowski, 1961).

The qualitative evidence from all these studies shows that, in terms of cell numbers, very large aggregates of identical cell types arise. This argues strongly in favour of some clonal growth without mixing and it is this circumstance which has prompted a number of attempts (Mintz, 1971; McLaren, 1972; Lewis, Summerbell and Wolpert, 1972) to analyse data in terms of the early history of the tissue in question.

The problem of drawing conclusions about the unobservable



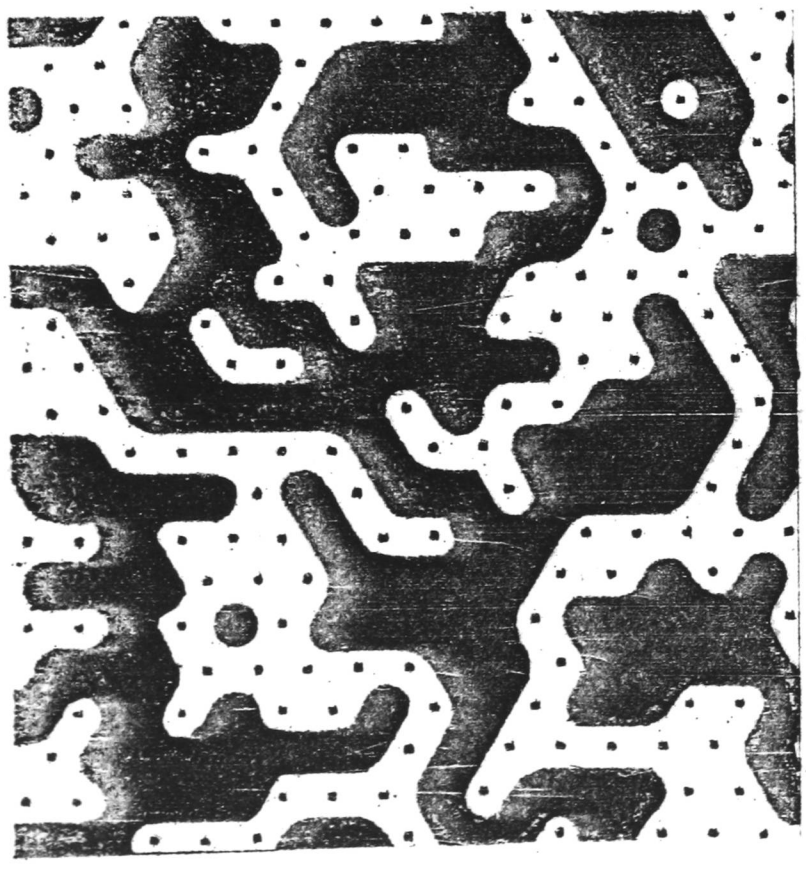


FIGURE 4.1 (a) Hexagonal two dimensional arrangement of 'cells' as considered in this chapter. Line L - L is a one dimensional row. (b) Computer printout of a section of a two dimensional array of hexagonal 'cells'. The array positions are printed as '.' or '*' to represent 'cells', and show A and B types of cells respectively. A proportion of 50:50 has been used, and all B cells have been blocked in as black areas for contrast.

early events from the evidence of the final outcome requires the investigation of models which have clearly defined assumptions and give quantitative answers in terms of which the experimental data can be interpreted. This paper considers the properties of a model system of proliferating 'cells' of two recognisable types, their aggregation into 'patches' of groups of identical cells and the distribution of 'patch sizes' in the system when proliferation has ceased. It then considers the problem of analysing biological material and what conclusions may be drawn from such an analysis.

THE MODEL.

Two special cases will be considered which are thought to have some embryological relevance. (1) a one dimensional row of cells; (2) a two dimensional array of hexagonally packed cells. In both cases we shall discuss mixtures of two recognisable cell types, A and B.

For the one dimensional case a patch is defined as a sequence of cells of one type bounded by cells of the other. For the two dimensional case, a patch is defined as an aggregate of cells of one type totally surrounded by cells of the other (Fig. 1a and b). The assumption, applicable to both cases, is that, for any given proportion of cells, there is random cell movement resulting in chance aggregates of identical cell types. A second stage is then considered when cell mixing has ceased and proliferation takes place which increases the number of cells (with or without synchrony) in the tissue without altering the relative position with

respect to their neighbours (clonal expansion). How far this abstract dichotomous behaviour corresponds to reality is a separate issue. We shall, however, suggest some criteria which bear on this question.

The problem can be resolved into a number of questions:

(i) for a population of 2 distinct cell types, A and B, in which the fraction of A cells is p, what is the probability of finding aggregates of cells (patches) of each type consisting of 1, 2, 3, .. cells?

(ii) how does the distribution of patch sizes depend on p?

(iii) how does the variability in rates of cell division affect the relationship of 'initial patch size' (before expansion) to 'final patch size' (after expansion)?

(iv) how does the absolute number of patches depend on the time at which mixing of the population ceased?

These are questions based on the theory of random clumping (see, for example, Roach, 1968). While an explicit algebraic solution is known for a one dimensional row, no complete solution has been obtained for a square 2 dimensional array, nor for the more realistic format for biological cells (Williams and Bjorknes, 1972; Autonelli, Rogers and Willard, 1973) of a close packed hexagonal array. Some analysis and computer study of such an array was therefore undertaken which yielded information on the questions posed above. (See also J. West, 1974).

The one dimensional row

Consider the distribution of patch sizes during random mixing. Let

N = total number of cells at a given time

p = probability that a randomly chosen cell is of type A (which, for large N, is the proportion of cells of type A), and 1 - p is the probability that it is of type B,

a = mean number of patches of type A.

n = number of cells in a specified patch in which all cells are of the same type, but bounded by cells of the opposite type (patch size),

S_n = mean number, and

s_n = mean proportion, of patches of size n in the array.

Unless stated to the contrary, n, S_n and s_n refer to A type cells.

The probability that a clump of A cells of, say, size n = 3 occurs, is that of having a sequence...BAAAB ... or $p^3(1-p)^2$.

In general, from

Roach (1968)

$$S_n = Np^n(1-p)^2 \qquad \ldots(1)$$

The mean number of patches of type A is

$$\underline{a} = \sum_{n=1}^{\infty} S_n = Np(1-p) \qquad \ldots(2)$$

and thus the proportion of patches of type A of size n is

$$s_n = S_n/\underline{a} = p^{n-1}(1-p) \qquad \ldots(3)$$

It is often more convenient to consider the mean number of patches per unit length (or unit number of cells). From (2) :

$$a/N = p(1-p) \qquad \ldots(4)$$

In these equations we assume that N is sufficiently large that

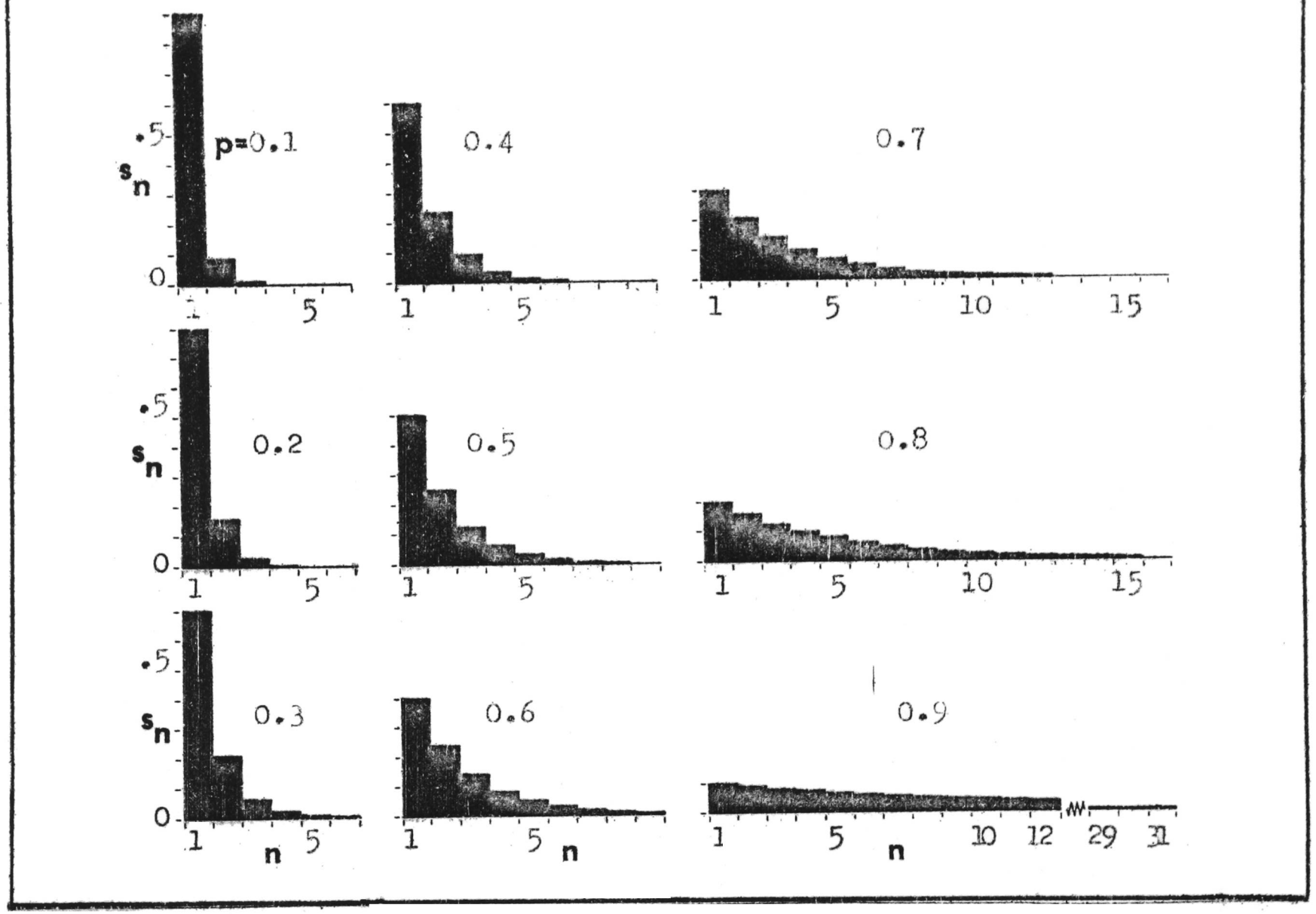


FIGURE 4.2 Histograms of s_n, mean proportion of patches of size n, against n for p = 0.1 to p = 0.9.

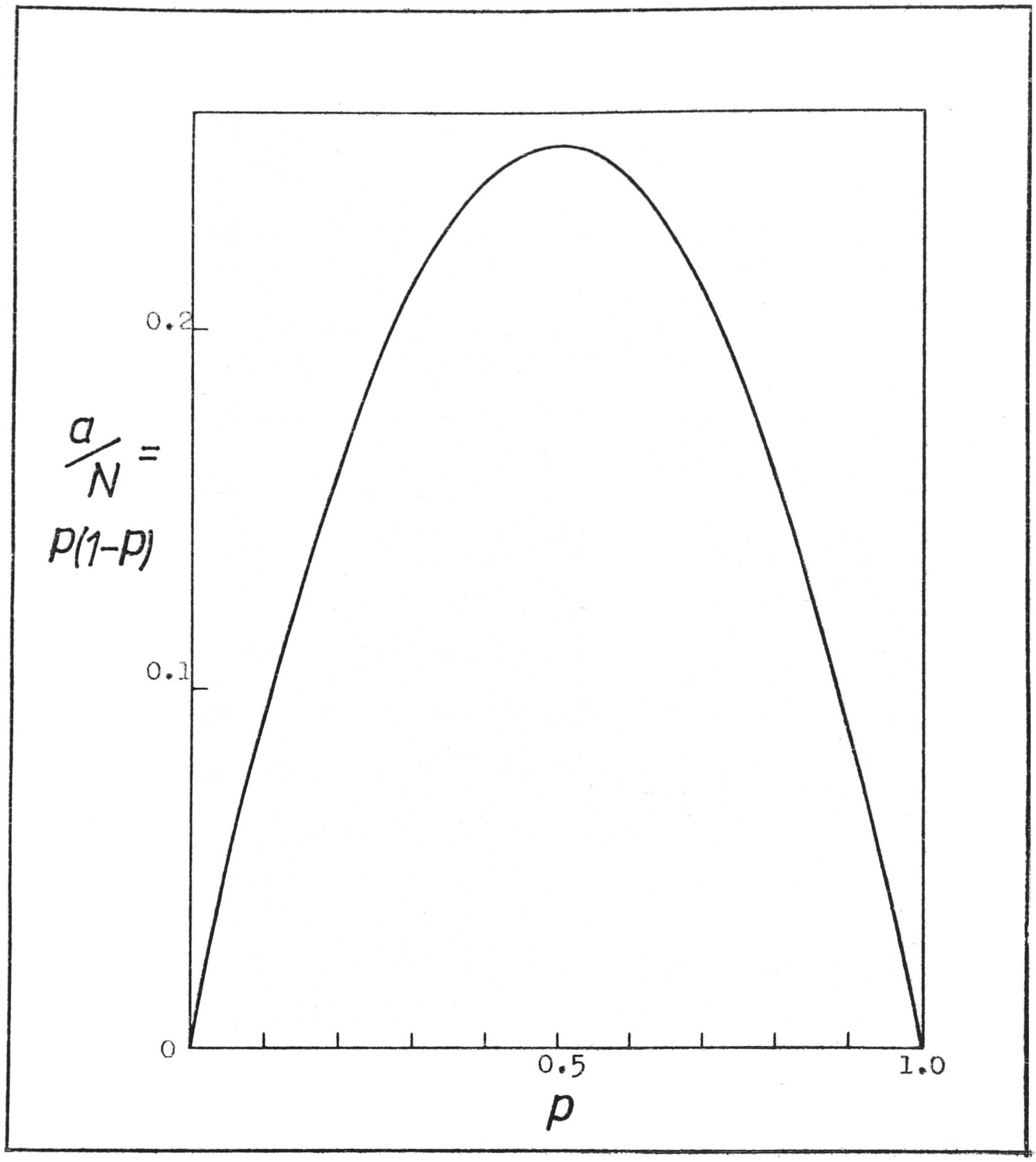


FIGURE 4.3 Plot of p(1-p), the mean number of patches of type A (a/N), against p, the proportion of cells of type A.

patches at the end of the row comprise a negligible proportion. The observed number of patches of type B equals that of A or differs by not more than 1; the mean number is the same.

Equation (3) allows us to calculate the distribution of patch sizes. This is shown in Fig. 2, for a number of values of p.

From equation (4) we see that the mean number of patches, irrespective of their size, for a given N, is at a maximum when the cell types are of equal frequency, and is symmetric about $p = 0.5$ (Figure 3). At this maximum there are expected to be one-quarter as many patches of one type as there are total cells in the initial array. At all other values of a/N, however, there are two corresponding values of p satisfying the relationship. The problem of chance variation in the total number of patches for a single array when N is small is discussed later.

The two dimensional array.

The same symbols will be used as for the one dimensional case, but with the addition of primes, e.g. S'_n denotes the mean number of patches of size n. It is possible to predict analytically the mean number of patches of small size, but for $n > 8$ the possible topological arrangements are so numerous that, even with the help of a computer, their enumeration is virtually impossible. Because S' cannot be calculated for all values of n, it is impossible to give an explicit value for the total number of patches expected and therefore for the proportions, s'_n, of patches of any size n. However, a useful measure is the relative frequency of patches of different sizes, given by S'_2/S'_1 for patches

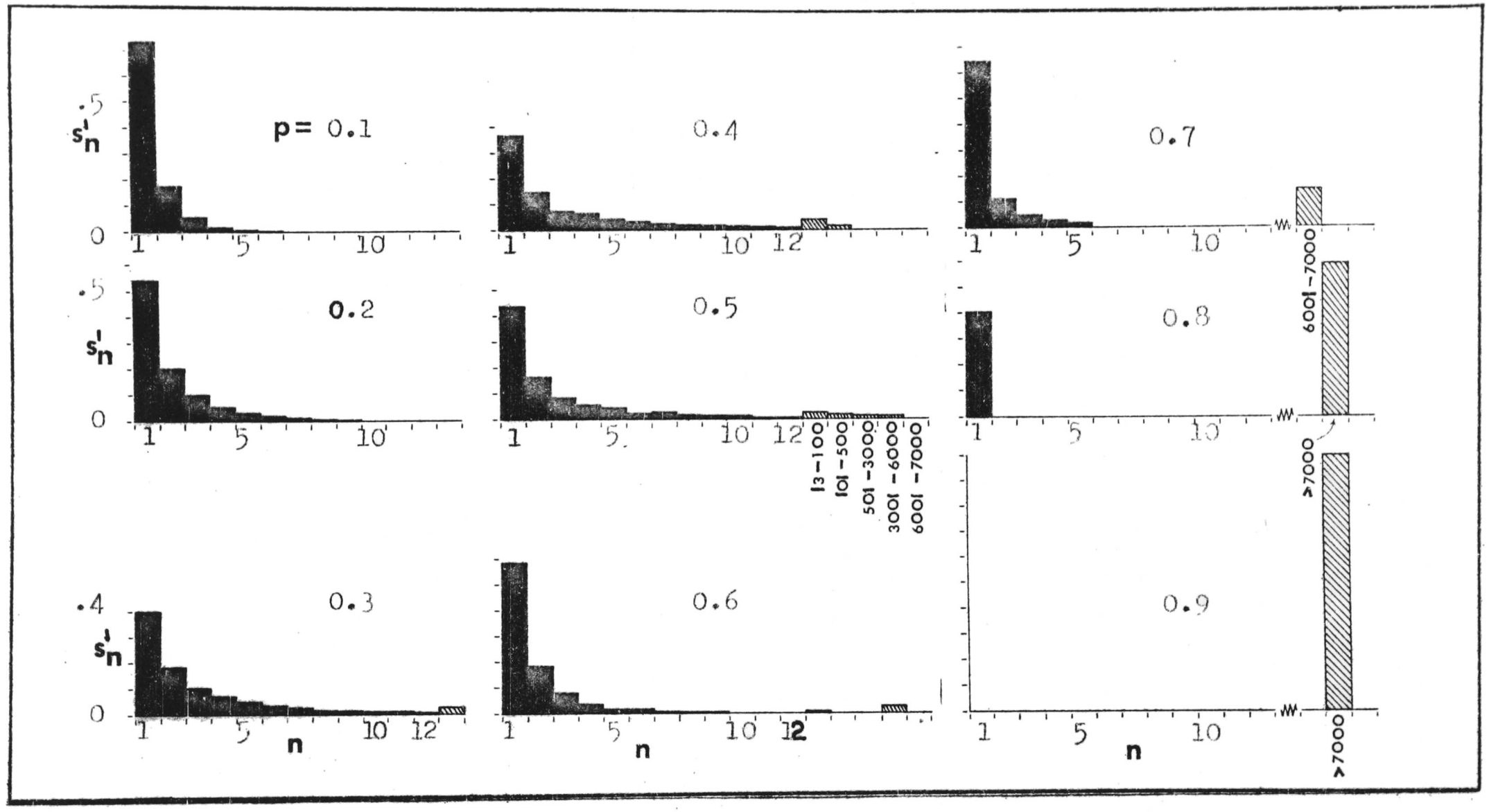


FIGURE 4.4 Histograms of s'_n, mean proportion of patches of size n, against n for p = 0.1 to p = 0.9. N = 10,000

of size 2 and 1, for example. Following methods given by Roach (1968) for square lattices, we find that for the hexagonal lattice, with N large.

$$S_1' = Np(1-p)^6, \qquad \ldots(5)$$

$$S_2' = 3Np^2(1-p)^8 \qquad \ldots(6)$$

giving $s_2'/s_1' = 3p\ (1-p)^2$. Further values of s_n'/s_1' are listed in the Appendix. It is thus possible to calculate for any p, the ratio of the proportions of the smaller patch sizes.

In order to overcome the analytical difficulties referred to above, a computer simulation was used to obtain approximate values for the complete distribution of patch sizes. In brief, the simulation was achieved by a program which would randomly assign positions to type A and B cells for any given value of p in a total number of two-dimensional array positions (N) varying between 100 and 10,000. The array positions were arranged in a hexagonal format. The program was designed to search for all the neighbours of equal cell type, designate such aggregates as 'patches' and record their number in the various size classes. Ten replicates were run for each set of parameters, and their means were used to construct Figs. 4, 5 and 6.

The patch size distributions shown in Figure (4) are similar to the one dimensional case, except that, as expected, the number of large patches increases much more rapidly with an increase in p. There are 6 neighbours to each cell and therefore the probability of having no neighbours of identical type becomes very small at $p > 0.6$.

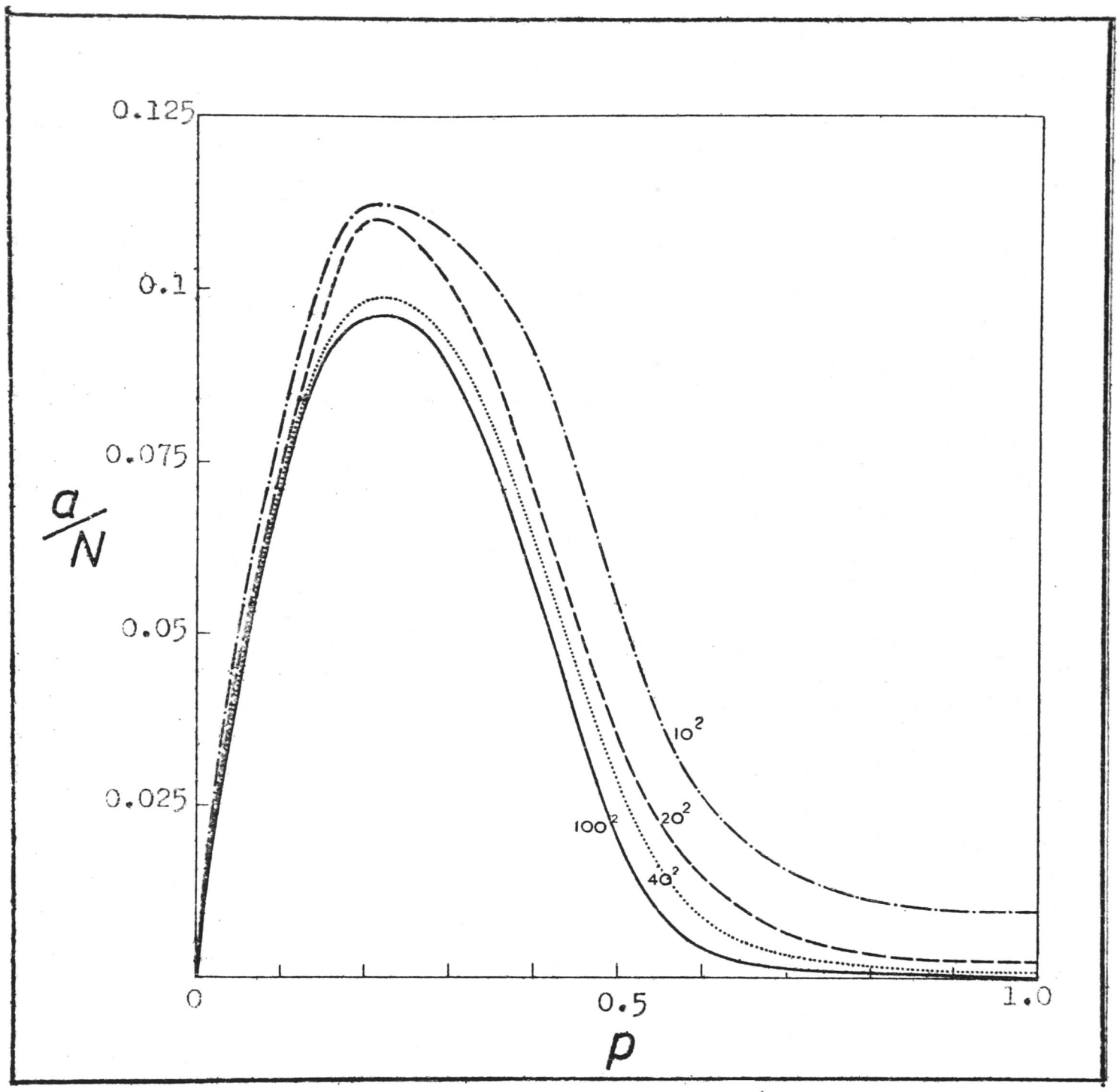


FIGURE 4.5 Mean number of patches of type A, a'/N, against p, the proportion of cells of type A, for a series of array sizes from 10*10 to 100*100.

The mean number of patches of type A, $\underline{a}'/N$, as a function p, is shown in Figure 5. Unlike the one dimensional case (Fig. 3) where end effects (adding or subtracting one patch to the mean) are negligible for almost all row lengths, the corresponding edge effects for small two dimensional array sizes must be taken into consideration. Figure (5) shows a family of curves which differ from each other by small but significant amounts. As can be seen, the $\underline{a}'/N$ values for the smallest array (100 cells) are larger for all p values. The array consisting of 10,000 cells approaches an infinite array. The 'excess' values shown for the smaller arrays must be attributed to edge effects. This is due to the failure of edge cells to be juxtaposed to like cells at the boundary of the array. (Whether this is a real phenomenon in living tissue or only a computer artefact depends on the early morphogenesis of each individual tissue. A spherical mass of tissue is unbounded and therefore would show no edge effects for all values of N. A flat sheet of cells, however, would correspond to the two dimensional computer array).

A different algebraic approach was used by Domb and Sykes (1961). They give an estimate of the mean patch size (M) as a power series in p for hexagonal and other lattices. The mean number of patches $\underline{a}'$ would then be given by Np/M and from their formula would be:

$$\underline{a}'/N = p/\left[1+3p+6p^2+12p^3+24p^4+33p^5+60p^6+99p^7+156p^8+276p^7\right] \quad \ldots(7)$$

The series is quite accurate for small p values, but as p increases, the mean patch size will eventually become infinite, i.e. a single confluent patch. Domb and Sykes estimate that the critical value

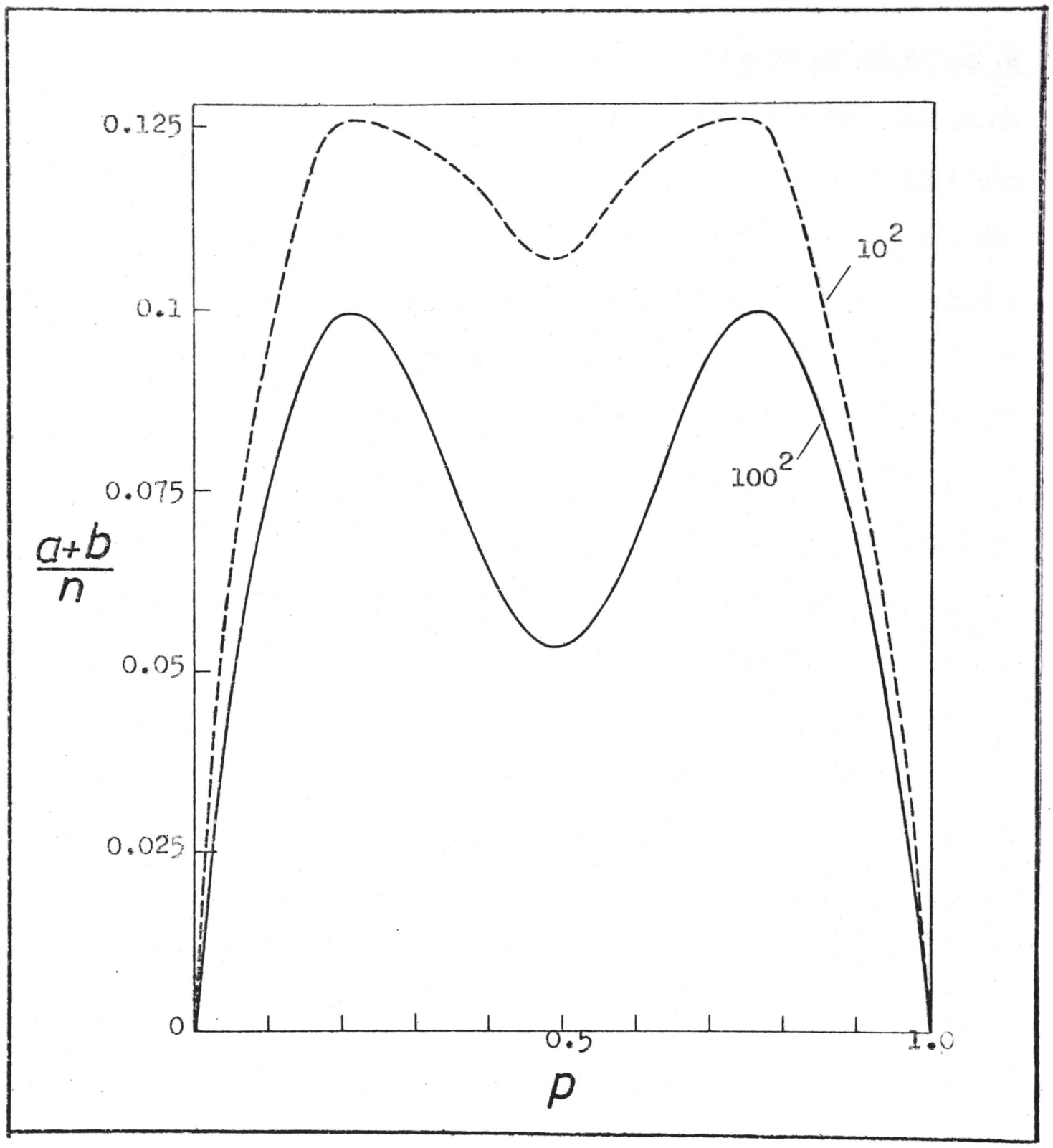


FIGURE 4.6 Overall mean number of patches a'+b'/N is symmetric about p=0.5. Array sizes 10*10 and 100*100.

at which this happens is $0.60 < p < 0.75$. These figures fit well with our computer simulation (Fig. 5).

The mean number of patches for the two dimensional array has its maximum at about p = 0.2. This asymmetry reflects the same probabilities of neighbour interactions which give rise to the distribution of fig. 4.

In the one-dimensional case the number of patches of the two types, in any one array, could not differ by more than one. The same tight relationship does not hold for the two-dimensional case. The mean number of each type, however, is entirely dependent on p and only when p = 0.5 will the mean numbers be equal. At small values for p, say at 0.1, A type cells will have $\underline{a}'/N \sim 0.075$ while for B type cells, $\underline{b}'/N \sim 0.000125$. In fact, the majority cell type, B, could in such a case, be a single confluent mass or patch.

The symmetry of figure (3), dealing with the one dimensional case, implies that if the number of patches of both types are counted, a plot of $(\underline{a} + \underline{b})/N$ against p will generate a curve with a maximum at 0.5 but with the ordinate being double that of figure (3). This is not the case for a two dimensional array where the same plot of $(\underline{a}' + \underline{b}')/N$ yields a bimodal distribution with an intermediate minimum at p = 0.5. This is shown in Figure (6) for two array sizes of 100 and 10,000 respectively. (It may be noted that the small array, having a considerable proportion of 'edge cells', has higher total number and a smaller difference in its intermediate minimum. In the limit, a very small array will tend to show the same behaviour as the linear row,

* By mixing is meant the process by means of which individual cells are free to change position in a random manner. This will in part be due to the absence of any cell-cell adhesion, morphogenetic movements and any perturbing forces present in the tissue. Changes in any of these properties could cause progenitor cells to remain in more or less the position of their 'birth'. Whether this takes place at one point in time or whether this change requires a finite period to establish itself is not known. What is clear, however, is that at some stage the cell population is growing clonally.

the latter consisting only of 'edge cells' without lateral neighbours.)

CLONAL EXPANSION.

So far we have considered how the mean number of patches and their size distribution depends on the proportion of the two types of cells, their total number and the geometry of the array. Our analysis allows for cell proliferation provided random mixing* continues during such proliferation. Other considerations enter, however, if there is a time point in development at which cell mixing ceases, and cell proliferation proceeds clonally. Under these conditions, absolute number of patches, a, and patch size distribution remains constant, but, since total cell numbers increase, a/N decreases. At the end of development it might, therefore, be thought that the patches in the tissue corresponded to the number and distribution of the cells at the time when clonal growth started.

If there was no variability in the rate of doubling, or all divisions were synchronous, the relative sizes of patches derived from 1, 2, 3,... cells would be exactly in the proportion 1:2:3:... and we could compute the degree of multiplication from e.g. the size of the smallest patches. In practice this is unlikely to be the case and we have to take account of 'noise' in the observed patches due to cell-to-cell variation in rates of division, and to variation in the ages of cells (and thus time of next division) after mixing stops. Assuming that there is no difference in the mean rate of division

among cell lines, and that the time at which any cell divides is uncorrelated with the division time of any of its 'ancestor', or 'progeny' or neighbouring cells, it is possible to derive a relationship between the variation in doubling time and that of patch size. An outline of the derivation is given in the Appendix.

Let γ be the coefficient of variation of doubling time, assumed to be small ($<<1$), then we can estimate c_n, the coefficient of variation of patch size, after clonal expansion of patches initially having n cells.

If cells are completely asynchronous at the time mixing stops it is shown in the Appendix that

$$c_n = (\ln 2) \left[2\gamma^2 + 1/12\right]^{\frac{1}{2}} n^{-\frac{1}{2}} \qquad \ldots(8)$$

approximately, where the term 1/12 arises from the initial asynchrony of the population. Thus, each discrete 'initial' size class gives rise to a 'final' distribution with overlaps between classes.

The distribution of final patch sizes after clonal expansion is obtained by combining the distribution of initial sizes (Fig. 2 for the one-dimensional case) with the distribution generated by the variation in doubling time. This is done for the one-dimensional case in Fig. 7 for a few values of the proportion of A cells (p) and coefficient of variation due to clonal expansion (c_n). If doubling time is normally distributed, patch size will be roughly lognormally distributed, and the latter assumptions is used for Fig. 7. a, b, c, d. (It may be appropriate to assume a log-normal distribution of doubling time,

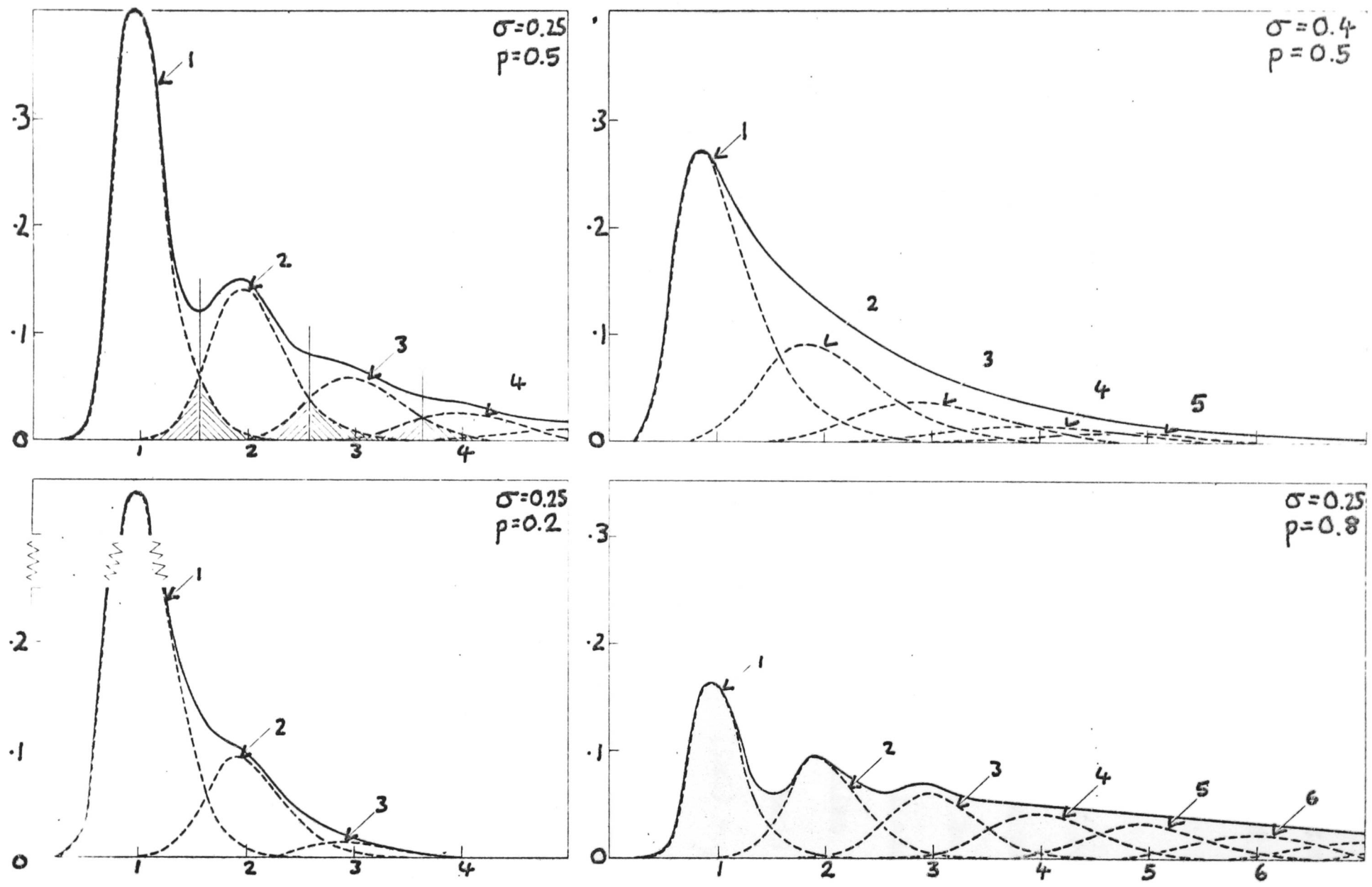


Figure 4.7. Distribution of final patch sizes after clonal expansion. Vertical axis: proportions of clones of the various sizes. Horizontal axis: number of cells from which clones are derived. Apices of dotted distributions for each size clones are marked with diagonal arrows. Overall observed distribution is solid line.

(Schmid, 1967) which would then induce a distribution of final patch sizes further skewed to the right.)

It will be noted that for small values of c_n, (e.g., 0.125, Fig. 7a) the distribution is quite discrete and identification of the size classes not difficult. This is, however, not so for higher values of c_n. For example, the distribution for the smallest class, arising from single cells, has the following coefficients (c_1) depending on the assumed values for γ.

γ =	0.1	0.2	0.3	0.4
c_1 =	0.22	0.28	0.36	0.44

Fig. 7b shows the distribution for $c_n = 0.25$, a not unreasonable value. The assignment of the proportion attributable to each size class is not directly possible. Given a distribution such as Figs. 7b, c and d it is nevertheless possible to extract the relevant information. For this an alternative formulation of eqn. (3) may be used (Slack, 1974). Taking logarithms of (3) we obtain:

$$\log s_n = n \log p + \log \frac{1 - p}{p} \qquad \ldots(9)$$

A plot of $\log s_n$ against n should give a straight line of slope $\log p$ and intercept $\log \frac{1 - p}{p} = \log \frac{q}{p}$, the logarithm of the ratio of the two types of cell.

A plot of any of the discrete frequency distributions in Fig. 2 would follow the above relationship as would also the continuous distributions in Fig. 7 provided the underlying component areas of each class are plotted (since they are, of course, the same proportions).

Since only the sum of the overlapping distributions (solid lines) are accessible, we outline a way of estimating the individual components. This is based on the fact, visible by inspection of Fig. 7, that the component distributions cross approximately half way between the peaks of the components. Furthermore, the area 'lost' to the preceding component is very nearly equal to the area 'gained' from that component. If, therefore, the interval is chosen at the half way points the areas so found will be very nearly the correct proportions of the components. To find the appropriate interval a first estimate will usually be possible by observing the two smallest classes (of n = 1 and 2 or any multiple of these) which will show a minimum between them. This interval is then taken as the 'scale' for dividing the remainder of the distribution into 'classes'. The resulting proportions are a first estimate of s_n values for equation (9). Further estimates can be obtained by taking small positive and negative increments to the interval in order to obtain a best linear fit to eqn. (9).

Before proceeding to the discussion of possible applications, we summarise the properties of the model.

(1) The distribution of patch sizes is characteristic of a unique value of p.

(2) The mean number of patches of one type of cell per unit row or array, $\underline{a}/N$, is characteristic of two values of p, the proportion of the type.

(3) The one-dimensional row and the two-dimensional array have different patch size distributions for the same value

of p.

(4) Variation in doubling times of the cells in proliferating clones after cessation of mixing changes the discrete classes into overlapping distributions, the overlap being the more pronounced the larger the patch sizes. With very large coefficients of variation the distribution approaches an exponential whose index is n log p (see eqn. 9).

APPLICATION TO EXPERIMENTAL MATERIAL.

We may now examine under what circumstances the model may be used to analyse biological material. We shall suggest what useful measurements can be made to answer two classes of questions, one concerned with identifying events in the early history of the tissue, and the other concerned with the assumptions of the mechanisms involved.

Almost all biological tissue is three dimensional, heterogeneous in cell types and irregular in terms of packing the cells. Most tissues can only be examined in section. How far does such a section differ from the abstract plane of hexagonally packed 'cells' on which this analysis is based? Even with a regularly packed tissue an arbitrary plane cut through such a three dimensional array will, only by an outside chance, pass through the centre of all cells (or patches). The average plane will be a 'glancing' one and the mean size of patches will be reduced. Irregular packing will have the same effect. Similarly, a line drawn through the centres of 'cells' (Fig. 1) (to which our one-dimensional row analysis applies)

will not have quite the same patch profile as when it is drawn in a random orientation. Since our suggested applications depend strongly on such a 'line analysis', we have examined graphically the deviation of the patch sizes and their distribution for a sample of lines drawn in a random direction over a hexagonal array. We find that the mean size of patches is about 84% of the 'true' patch size. Since, however, we found almost no overall correlation between the deviations of adjacent cells (although there are short runs with both positive and negative correlations) the relative proportion of patch sizes as shown in Figs. 3 and 7 will not be greatly affected. However, the variation in patch size is increased; the coefficient of variation in the length of cell cut by a randomly chosen line is about 0.25.

There are no parameters, except total cell number which will be discussed later, obtainable from a two-dimensional analysis that cannot be obtained from a one-dimensional study carried out on the same array. In view of the analytical difficulties referred to before and because of the much greater experimental advantages, we shall from now on deal solely with a 'line analysis' in terms of the one-dimensional model.

The procedure is, therefore, as follows:

Sections of tissue are taken and the two cell types identified by some suitable method. Random lines are then taken across the sections and the lengths of the two types of tissue measured in some arbitrary unit. The patch sizes so obtained are ranked and the frequency of their size classes are

plotted to give a profile as in Fig. 7, but as a histogram. The number of lines taken to give reliable values will depend on the size of the section in relation to the patch sizes, but there will be no benefit in taking many lines on the same section or many sections from the same tissue if the same patches are repeatedly sampled, i.e. if the total number of patches in the tissue is small. Both ordinate and abscissa are now transformed as follows. The absolute frequencies of the ordinate are divided by the total number of patches counted to give the proportions, s_n. The abscissa is 'scaled' into units corresponding to patches derived from one cell in the manner described in the previous section. We can now proceed to use these experimental data to address ourselves to a number of questions.

Homogeneity of development.

The implied or explicit assumption of many discussions of chimaera or X-chromosome inactivation is that the tissue examined is homogeneous with respect to its developmental history, i.e. that the examination of any part, apart from sampling error, reflects the whole. It may, however, be asked whether every part of, say, a liver has undergone the same average number of divisions as any other part. A second assumption is the identity of the two cells apart from their 'markers'. There may, however, be differential proliferation or viability of the two types of cells.

Our analysis enables us to examine these assumptions. Homogeneity implies that a unique value of p is obtained by each

of three methods. For convenience we will designate the operationally distinct estimates by different subscripts. They are, however, all estimates of the proportions of type A cells.

(1) The measurement of the proportion of two types of cells (or of the areas of patches) irrespective of size or number of patches (p_1).

(2) The value of log p, and hence p, obtained from the distribution of patch sizes of type A, using equation (9) (p_2).

(3) The value of log q (and hence p) obtained from the distribution of patch sizes of type B (p_3).

Homogeneity of development will be indicated if all three methods yield the same value of p for all domains of the tissue. Some qualitative conclusions may be drawn from significant deviations of this. We shall examine a number of extreme cases.

(i) There is spatial homogeneity but proliferation rates (or viabilities) of the two cell types are different. - Consider a system starting with equal numbers of two types, i.e. p = 0.5. Proliferation, unequal for the two types, with mixing will result in a distribution of, say, p = 0.7. At this point all three methods would give the same value. If mixing then ceases, the patch size distributions of the two types will be 'frozen' at p = 0.7 and q = 0.3 respectively but, because of continued differential multiplication, the overall proportions at a later time point (determined as p_1) will be much more extreme:

$p_1 \neq p_2 = p_3$.

(ii) There is homogeneity but the expression of one phenotype

is dominant relative to the other. - This applies particularly to X-chromosome inactivation where, it is assumed, there is a period when both chromosomes are 'active'. In the case of heterozygous pigmentation differences, black (B) is dominant to albina (A) and therefore all cells will be black until the random 'segregation' due to inactivation occurs, $p_2 < p_3 \neq 0.5$. The evidence suggests that melanogenesis begins quite late. In that case we would expect $p_1 = p_2 = p_3$. If pigmentation is not entirely cell-autonomous, however, we would, because of dominance, except $p_1 < 0.5$. The albino distribution will be truncated with fewer small albino patches resembling $p_2 > 0.5$ while the black distribution will tend to give $p_3 > 0.5$ also.

(iii) Cell proliferation is unequal in different parts of the tissue, but equal for both types. - It is clear that in such a case $p_1 = p_2 = p_3$ in every domain, but the absolute sizes of the various classes taken from one domain will not be the same as from another domain. If all patches are ranked together this will have the effect of swelling the larger classes in <u>both</u> types: $p_2 \neq p_3$. p_1 will lie between these values and will be the true value for the tissue.

<u>Epidermal Patterns in Mammals.</u>

The skin or pelt is a special tissue because of its characteristics as well as for its obvious histological importance as a source of data on colour patterns. There is good embryological evidence that the cells of the pigment tissue derive from a thin line of cells, possibly a single row on either side of the dorsal midline. The one-dimensional row therefore seems the appropriate model

without sectioning the line projections. 'Stripes' would be generated which are simply lateral extensions of the row (Mintz, 1971). Wolpert and Gingell (1970) have shown that for chimaeric mice on the assumption of p = 0.5 and a row of 32 cells on each side of the dorsal midline, a pattern of 'stripes' would be generated with a high probability of 8 of either kind, and a modal number of 17 stripes in all, which is a number often observed. The mechanism of stripe formation is assumed to be proliferation and invasion of melanoblasts in the growing skin. It is, however, significant that striping is not the invariable result (Mystkowska and Tarkowski, 1968; McLaren and Bowman, 1969), and that patches are an equally frequent occurrence. This indicates that the clonal coherence of lateral growth may not be as rigid as Wolpert and Gingell's analysis suggest. If some cell mixing takes place during the lateral proliferation in the skin, then although embryonically the tissue is derived from a single row, the pattern distribution will tend to a two dimensional array.

Taking random lines across the pelt may therefore be a more appropriate method. This would allow the use of all chimaeric material rather than only those individual mice where, by chance, lateral proliferation has been minimal.

It would be instructive to investigate how far the assumption p = 0.5 corresponds to the expected size distribution for this value. There is in fact little information on proportions of two types of cells. It has been usual to assume that, since chimaera arise experimentally from the aggregation of two 8 cell embryos of genetically distinct types, a p value of

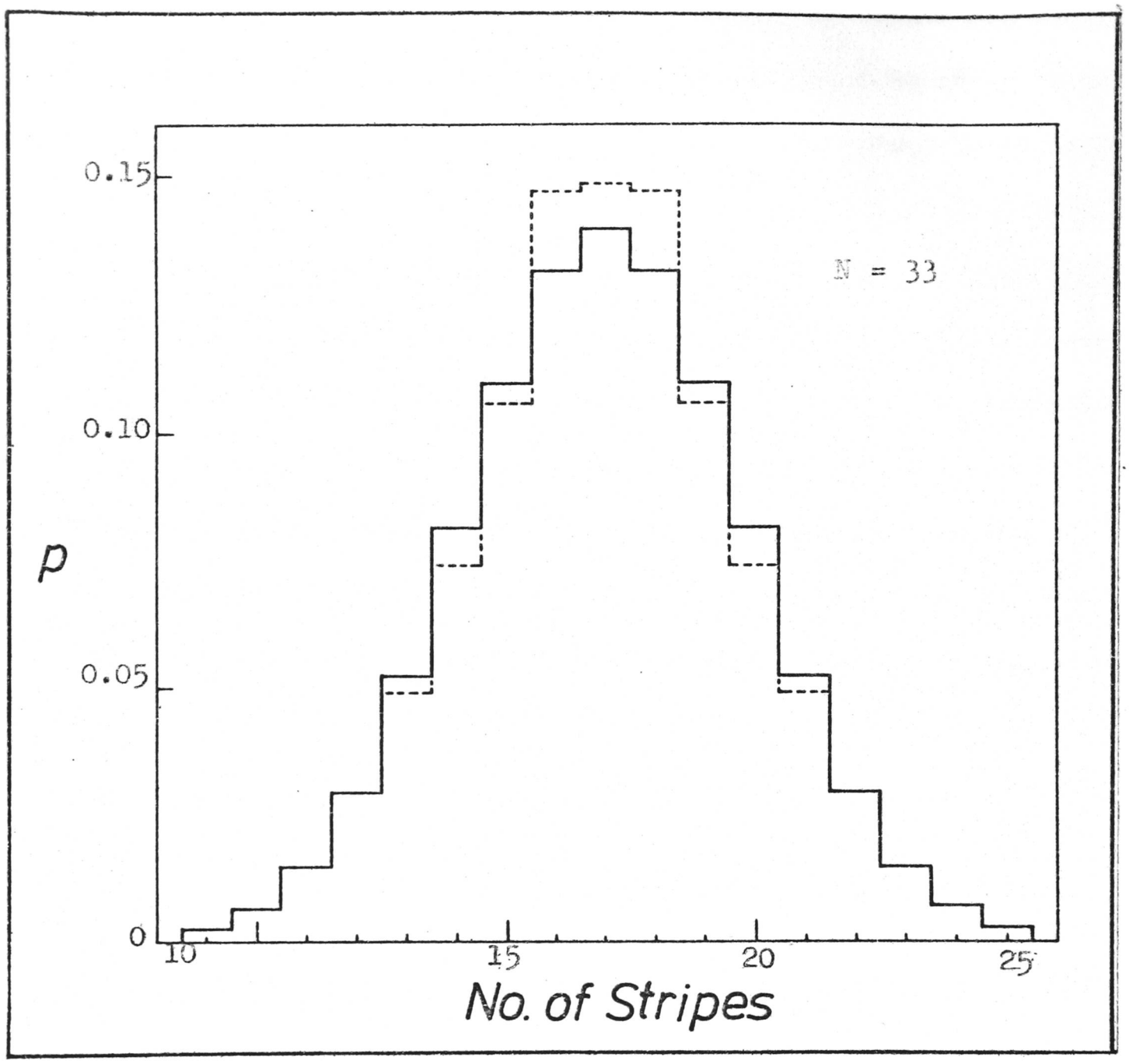


FIGURE 4.8 Probability distribution for the number of 'stripes' when 16 A and 16B cells are placed randomly in a linear sequence. Dotted expectations from Wolpert and Gingell (1970). Solid lines are our expectations. (See text for explanation of difference.)

0-5 is appropriate. It is, however, clear that the line of cells which gives rise to the skin is subject to sampling of the initial input ratio of equal numbers of two types of cells. By the time the neural crest cells are set apart to form the row of primordial cells the latter constitute a small fraction of the whole cell population. The proportion of two types of cell samples will then be binomially distributed, provided they have been randomly mixed. This distribution must be multiplied by the probability of distributions of patch numbers for any given proportion which Wolpert and Gingell considered. For an input fraction of p = 0.5 an explicit expression for the combination of both probabilities is given by the binomial distribution.

$$P(M) = \frac{(N-1)!}{(M-1)!\ (N-M)!\ 2^{N-1}}$$

where $M = a + b$, $1 \leqslant M \leqslant N$.

The distribution, for a mode of 17 patches generated by 33 cells, is given in Fig. 8. It can be seen that it differs slightly from that given by Wolpert and Gingell, which ignores the sampling distribution. It is by no means certain that there is a constant primordial number.
Variation in this would further spread the probability distribution of Fig. 8. An analysis of a series of chimaeric animals in terms of the experimentally determined number of patches should correspond to such a distribution. Within each chimaera the patch size distribution should correspond to one of the appropriate profiles.

Estimation of primordial cell number.

Provided consistency of p estimates indicates that the clonal expansion model is applicable, it is possible to estimate the number of cells at the stage at which mixing ceased and clonal proliferation began. Figures 3 and 5 for one and two dimensional arrays give the mean number of patches for specified p and a given number of primordial cells, expressed as a/N. Inverting this relationship, we can obtain an estimate of the number of primordial cells, N, by dividing the observed number of patches of type A by the a/N value appropriate for the observed proportion of A cells, p_1. To estimate the number of primordial cells in a three-dimensional tissue it will be necessary to estimate numbers of cells on a minimum of two sections (which would have to be perpendicular) and from that compute a "colume" or total number of cells. More sections would be necessary in irregularly shaped tissues.

Although, in chimaeric mice both cell types are present ab initio, the case of X-inactivation mosaics raises an additional problem. If clonal expansion has started before X-inactivation generates determined cell types, the calculated N number will be a true reflection of the time of this determination. If, on the other hand, cell mixing continues after the determination event, the calculated N number will 'overestimate' the time (in terms of cell numbers) at which inactivation occurred and will in fact give the time at which mixing ceased.

Concluding remarks.

How far this analysis will be useful in analysing biological material will, to some extent, depend on the experimental difficulties encountered. It is, however, suggested that considerations of patch size distributions apart from the modal number of patches or stripes should give further insight into the mechanism of pattern formation.

APPENDIX (i) Patch size frequency in the two dimensional model of a hexagonal array of closely packed cells.

The expected number of patches of size n is given by

$$S'_n = Np^n \left[\sum_k r(n,k) q^k \right]$$

where k is the number of neighbours for the particular configuration of the patch, and r(n,k) is the number of cell arrangements with k neighbours for n cells (Roach, 1968). Values of r(n,k) are listed in Appendix Table 1.

For example, with n = 4

$$S'_4 = Np^4(3q^{10} + 12q^{11} + 29q^{12})$$
$$= Np^4q^{10}(3 + 12q + 29q^2)$$

and

$$s'_4/s'_1 = S'_4/S'_1 = p^3q^4(3 + 12q + 29q^2)$$

The total number of arrangements for given n, i.e. $\sum_k r(n,k)$, increases by a factor of about 4.5 for unit increase in n ($n > 4$).

APPENDIX TABLE 1 No. of possible arrangements r(n,k) with k neighbours of patches of size n.

k/n	1	2	3	4	5	6	7	8
6	1							
7								
8		3						
9			2					
10			9	3				
11				12	6			
12				29	21	14	1	
13					66	43	30	6
14					93	153	111	69
15						298	366	291
16						306	840	957
17							1290	2349
18							1014	4299
19								5310
20								3408
Total	1	3	11	44	186	814	3652	16689

APPENDIX (ii) Relation between coefficient of variation of doubling time (γ) and coefficient of variation of final patch sizes for patches deriving from n cells (c_n).

Consider cell lines originating from a single cell. Let the mean doubling time be τ and its variance V(t). If there is variation only in the time of first division, which occurs at time t_1, and thereafter there is exponential growth at a rate α, the number of cells (N) at time $T \gg \tau$ is

$$N = 2\, e^{\alpha(T-t_1)} \qquad (1a)$$

If t_1 has a small coefficient of variation, from Taylor's series

$$V(N) = \left(\frac{dN}{dt_1}\Big|_{t_1=\tau}\right)^2 V(t_1) = 4\, e^{2\alpha(T-\tau)}\alpha^2 V(t_1) \qquad (2a)$$

approximately. But α and τ are related by $\alpha = (\ln 2)/\tau$ since $e^{\alpha\tau} = 2$,

so the coefficient of variation of N is

$$CV(N) = V(N)/e^{2\alpha T}\ \tfrac{1}{2} = \gamma \ln 2 \qquad (3a)$$

where $\gamma = [V(t_1)]^{\frac{1}{2}}/\tau$ is the coefficient of variation of t_1. Now continue the argument by allowing both the first and second pair of divisions (at times t_2 and t_3) to show independent variation. Eq(1a) is replaced by

$$N = 2\, e^{\alpha(T-t_1)}\,(e^{-\alpha t_2} + e^{-\alpha t_3})$$

Then using an extension to (2a) we obtain the extension to (3a),

$$CV(N) = \gamma\,(1 + \tfrac{1}{2})^{\frac{1}{2}} \ln 2$$

Taking account of all divisions, we get

$$c_1 = CV(N) = (1 + \tfrac{1}{2} + \tfrac{1}{4} + \tfrac{1}{8} + \ldots)^{\frac{1}{2}} \ln 2 \qquad (4a)$$

$$= \gamma\sqrt{2} \ln 2 = 0.98\,\gamma \approx \gamma$$

Notice that $\frac{1}{2}C_1^2$ is contributed by the first division, $\frac{1}{4}C_1^2$ by the second and so on. Thus, if the first division is synchronous, C_1^2 is halved.

If adjacent cells divide independently, both the mean and the variance of the number of cells in the final patch will be proportional to n, the number of cells in the initial patch. Therefore,

$$C_n = C_1/\sqrt{n} \qquad (5a)$$

Asynchrony of divisions at the time cell mixing stops will introduce additional variation in the time of first division, t_1. Complete asynchrony would give a uniform distribution of cell age which adds a square coefficient of variation of 1/12 to the variation contributed by the time of first division. Hence, with complete asynchrony, from (4a) and (5a)

$$C_n = (\gamma^2 + 1/12)^{\frac{1}{2}} \ln 2 \qquad (6a)$$

As with the other results, (6a) is approximate.

CHAPTER FIVE

COMPUTER ANALYSIS OF DIVISION PATTERNS IN THE DROSOPHILA EYE DISC

INTRODUCTION.

The active life of the fruitfly Drosophila melanogaster is divided into three distinct developmental phases; larva, pupa, and imago. During the larval stage, cells set aside in the embryo develop as 'imaginal discs', destined to form the surface structure of the adult insect, with separate imaginal discs for legs, wings, eyes, and so on. A characteristic feature of the discs is that they are all initially comprised of a single columnar epithelial tissue layer. This layer remains associated throughout development with a membrane covering made up of a non-cellular basement lamina on its underside, and a cellular 'peripodial membrane' above.

The morphogenesis of the various adult organs produced from the discs has been studied (for example by using genetic mosaics, produced by genetically 'tagging' single larval disc cells after varying periods of development. This can be done by induction of mitotic recombination, by position effect variegation, or by the use of gynandromorphs. If the fly is heterozygous for a recessive marker gene (affecting, for example, specific pigment colour or bristle shape), individual cells can be made to give the homozygote (or hemizygote in the case of X-linked gynandromorphs) genotype, thus exposing the marker trait. The size of the clone resulting from the marked cell can be used in the adult as a means of finding the number of cell divisions that have occurred between

induction of the marked cell and adulthood; to find the number of cells that were in the disc at the time of tagging; and to discover the time that the various determination steps are set up during development.

By tracking a particular cell lineage in this way, the shape of the resulting clone can be analysed in the adult, and this information can be used to find out the division patterns of the cells which make up the clone. Many examples of the use of this and other techniques in Drosophila exist in the literature (see, for example, Postlethwaite and Schneiderman, (1971), antennae; Becker, (1957), eye; Bryant, (1970), wing). These workers have shown both that 'directed division orientations' occur frequently in Drosophila larval imaginal discs, and that the boundaries of the clones are coherent: for each marked cell there is normally only one clone seen in the adult. The latter observation indicates that cell migrations are probably not seen to any great extent in imaginal discs.

These experiments do not show, however, the mechanism by which cells co-operate to enable the various discs to undergo morphogenesis. Morphogenesis is one of the most important and least understood areas of developmental biology because it is so difficult to visualise how large populations of cells can form themselves into such ordered and region-determined structures as imaginal discs. To help surmount this problem, a computer model has been produced to simulate growth in a particular disc, that of the head/eye. This disc was chosen because of the wealth of data on clone shapes in the adult eye, and also because these

clone shapes suggested that cell division in the eye part of the disc took place in certain directions. (Becker, 1957).

It is shown that 'tagged' computer 'cells', dividing randomly within a larger 'cell mass', representing the 'eye disc', can give orientated clones similar to those seen by Becker (1957), and by Baker (1967). The conditions needed to generate the computer 'cell clones' are array boundary constraints biasing division in certain directions; the constraints are analogous to contact with membranes or surrounding organs in vivo. The findings suggest that such constraining activities are of importance in the morphogenesis of the Drosophila eye disc. The physical nature by which these constraints might be acting in the larva is discussed. Some possible applications of membrane constraint mechanisms in other discs, and in a more general context are also discussed.

PREVIOUS EXPERIMENTS.

By using the technique of somatic crossing over, Becker, (1957) has produced X-ray induced cell clones in the Drosophila eye. Larvae were irradiated at various times during the early development of the eye disc, the use of particular eye pigment mutants allowing observation in the adult of clones arising from cells undergoing mitotic recombination. As Becker's flies were heterozygous for the alleles w (white), and w^{co} (coral) and had eye pigment of an intermediate colour to the two homozygotes, mitotic recombination produced both w/w and w^{co}/w^{co} type cells, giving rise to a 'twin spot'. The clones produced by both cell

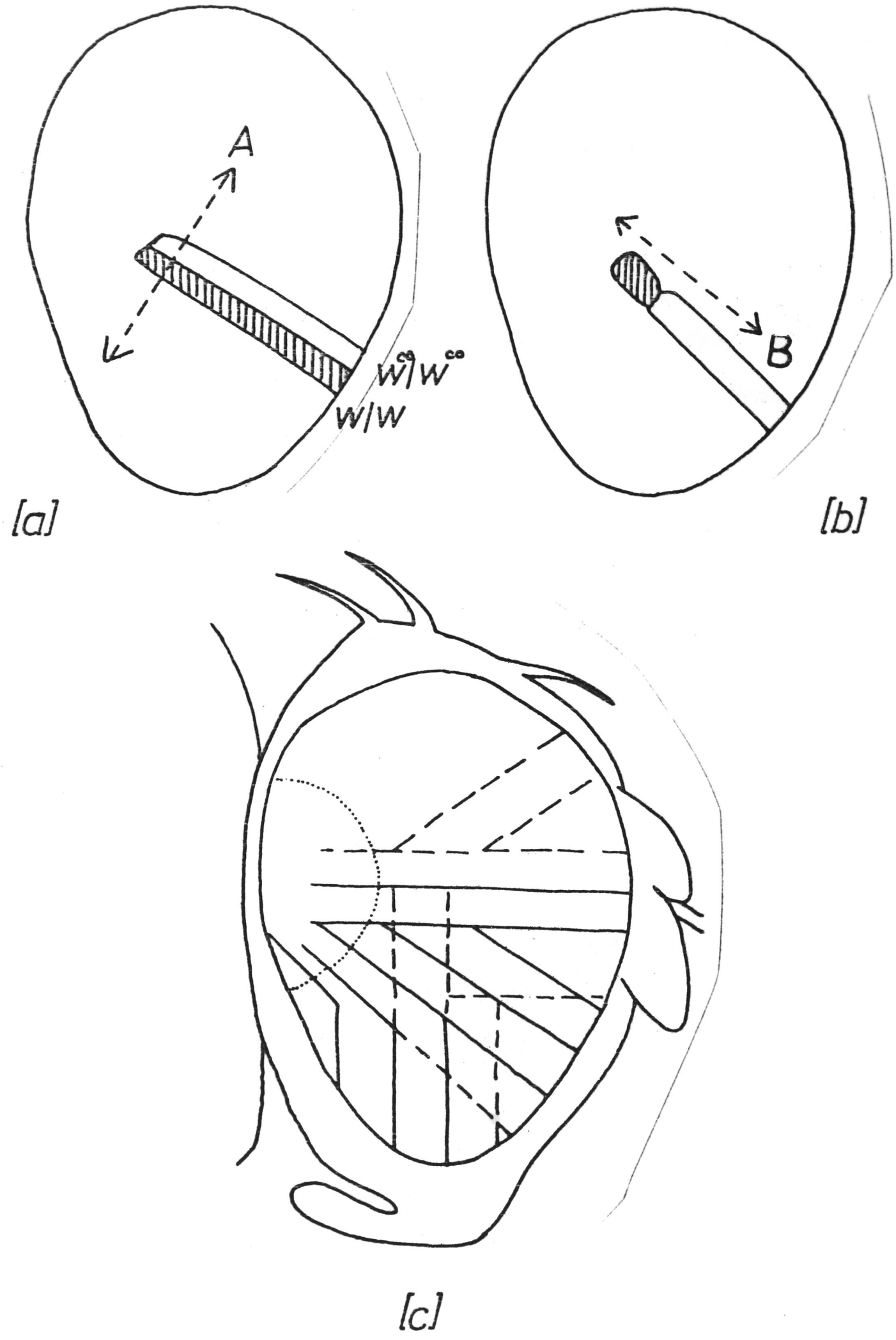


FIGURE 5.1 Eye pigment clones in adult Drosophila eyes.
(a) Clone produced in the 1st larval instar. First divisions occurred along axis A, and subsequent divisions along axis B.
(b) Second instar produced clone. All divisions along axis B.
(c) Becker's scheme of division sectors in the eye.

lines could be observed in the adult fly merely by looking at the eye pigment colouration differences.

From the shape of the clones in the adult eye, Becker concluded that the cells divide along two distinct axes during the time of positional organisation in the larval disc. The first orientation (figure 5.1a), only occurs up to the end of the first larval instar, and is along a roughly dorso-ventral axis, any 'tilt' depending on the dividing cells vertical position in the eye. At the end of the first instar, a 90° change in orientation takes place (figure 5.1b), and all subsequent cell divisions are more or less along the anterio-posterior axis.

By tracing a large number of the 'twin spot' patterns, and superimposing the drawings, Becker saw that the clones tended to occur in a number of defined sectors (see his scheme in figure 5.1c), reaching from the posterior to the anterior edge of the eye. As the cells in the twin spot clones induced prior to the end of the first larval instar (before about 24 hours of larval development) did not divide along the anterio-posterior axis until this time, Becker has suggested that the concomitant connection made by the head anlage with the larval brain at about the same time, may somehow result in the new orientation of eye disc cell divisions. (Becker, 1966).

In Drosophila, two imaginal discs form the compound eyes, head chitin, and attendant bristles of the adult fly. The discs are formed from an evagination of the embryonic pharynx, known as the peripodial sac. At the beginning of larval development, there are about 20 cells to each disc, and

exponential growth increases the cell number to about 3000 by the third larval instar. At about 72 hours of larval development, the onset of the third larval instar, ommatidial differentiation starts. The exact time and starter mechanism for this differentiation is not clear, but Steinberg (1943) provides evidence of 4-cell clusters visible in transverse sections through the ommatidial region at the 72 hour stage. Other authors have not observed this early aggregation (see for example, Waddington and Perry, 1960), although it is generally agreed that by the end of the third larval instar, the beginning of ommatidial differentiation is discernible. This differentiation takes the form of local aggregation of the presumptive ommatidial cells into goblet-like structures (Waddington and Perry, 1960). The major part of the process that eventually gives rise to the 30 or so specialized cells of each ommatidium takes place during pupation, and although no direct evidence exists, it seems reasonable to suppose that an individual ommatidium is either formed from a single determined 'stem cell' which subsequently divides, or from clustering of unrelated neighbouring cells.

COMPUTER MODEL.

The model consisted of a two-dimensional array with the array elements placed in a hexagonal format, as described in chapter three. Into the program was embedded a routine calculating the area of an ellipse of given radii . This ellipse, of variable shape, was superimposed onto the two-dimensional array, and was 'grown' in steps to represent the membrane

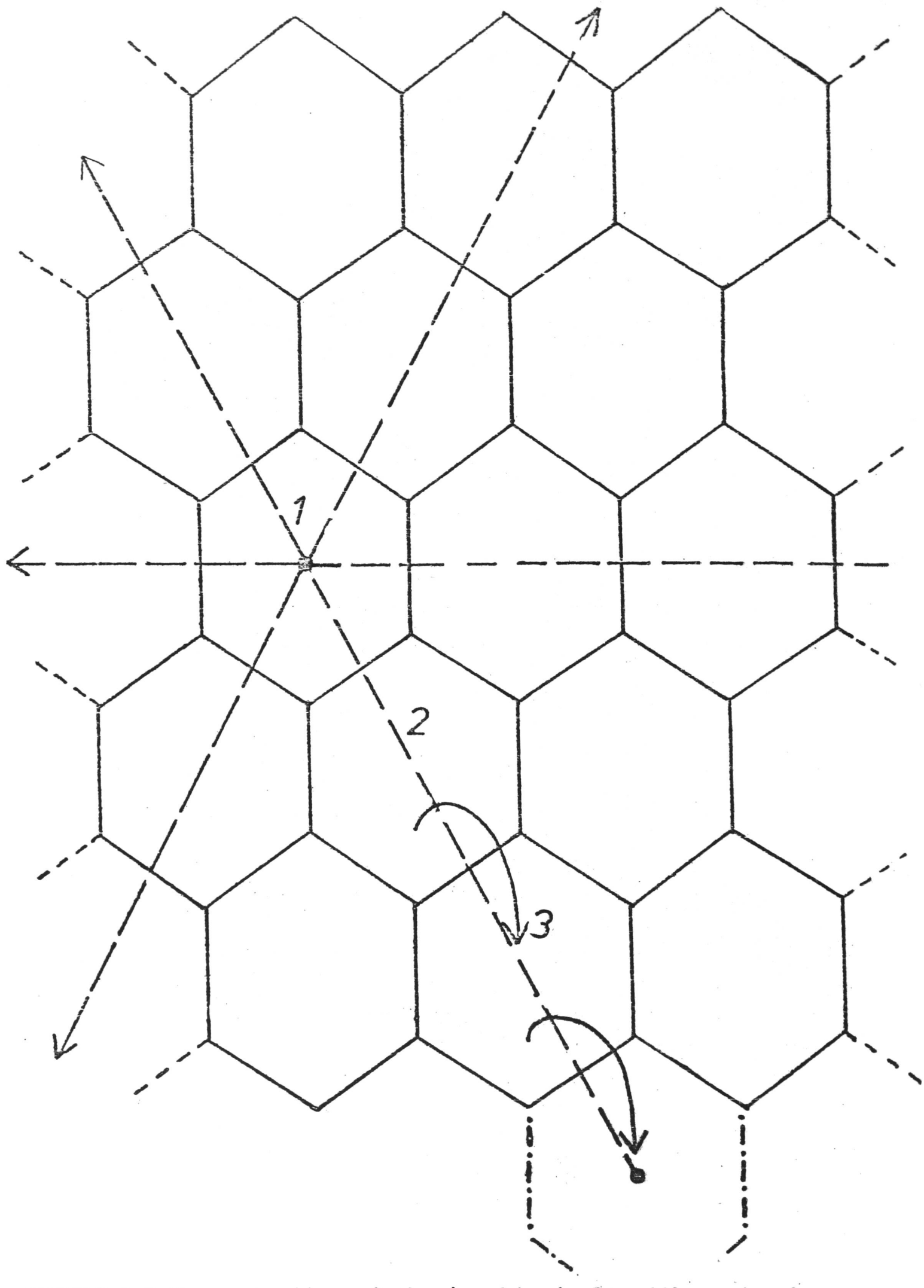


FIGURE 5.2 Demonstration of the 'pushing' algorithm using hexagonal cells. Cell 1 is to divide. It decides which is the nearest edge, in this case marked by cell 3. Cell 3 moves out one position, as does cell 2. The space occupied by cell 2 is hence cleared for the new daughter cell.

surrounding the growing 'eye disc'. As the eye disc itself grows in two-dimensional fashion, the model does not have such great 'dimensional limitations' as those encountered by other array-bound computer models of developmental systems (e.g. Ede and Law, 1969).

At the time the simulation was started, a variable number of starting cells making up the 'eye disc primordium' were placed inside the initial ellipse shape. Each cell was represented on the computer by an array element containing a numeric code differing for each cell type. The ellipse and cell mass were then 'grown' according to the growth parameters chosen; these parameters governed the direction in which the ellipse could 'grow', and the area of the ellipse necessary to be filled before it grew. The growth increment was also set - incremental growth was necessary because of the discrete nature of the simulation.

'Cell division' took place by each cell being 'pointed to' in turn (all cells having a chance to divide once before a fresh generation was started), and the chosen cell then attempting to divide at random into one of the surrounding six sites. If all six locations were already filled by cells, the following procedure was used. The locations in each of the six possible division directions were recursively searched by moving out through the cell mass until a free site was found: all the cells present in the line between the cell to divide and the free location then moved outwards one location in this direction. The daughter cell could then be inserted in the free space (see figure 5.2).

If any of the division directions were constrained (that is, impinged on the growing edge constraint), the cell could not divide in that direction. If all directions were constrained, no division took place, and the next cell to be processed was chosen at random. Cell division was allowed to proceed until about 730 cells were present in the disc, although some runs went up to 2,500 cells. It was found that little change in clone position, shape, and relative size occurred between a computer 'disc' size of 730 cells and the roughly threefold increase in size represented by the larger 'disc'. The smaller array was much more economical in computer time (each run taking 1/12 of the central processor time of the larger version) and hence many more simulations could be run.

There is no direct evidence regarding the number of ommatidial precursor cells present at the time 730 cells are present in each head disc. Becker (1957) suggests from his twin spot work that the eye part of the disc originally derives from two cells, whilst Bryant (1967) calculated that there are 13 progenitor cells in the whole eye/antennal disc complex (data obtained from gynandromorph studies). A similar technique was used by Postlethwaite and Schneiderman (1971), who estimated that there are 7 - 9 cells in the primordial antennal disc. The head disc, if these data are taken together, would start off with a ration of between 2/2 and 2/4 cells representing ommatidia/head chitin, but such small numbers are probably not very reliable. There are more cells per unit area in the adult ommatidia than in the head chitin, so there must be more

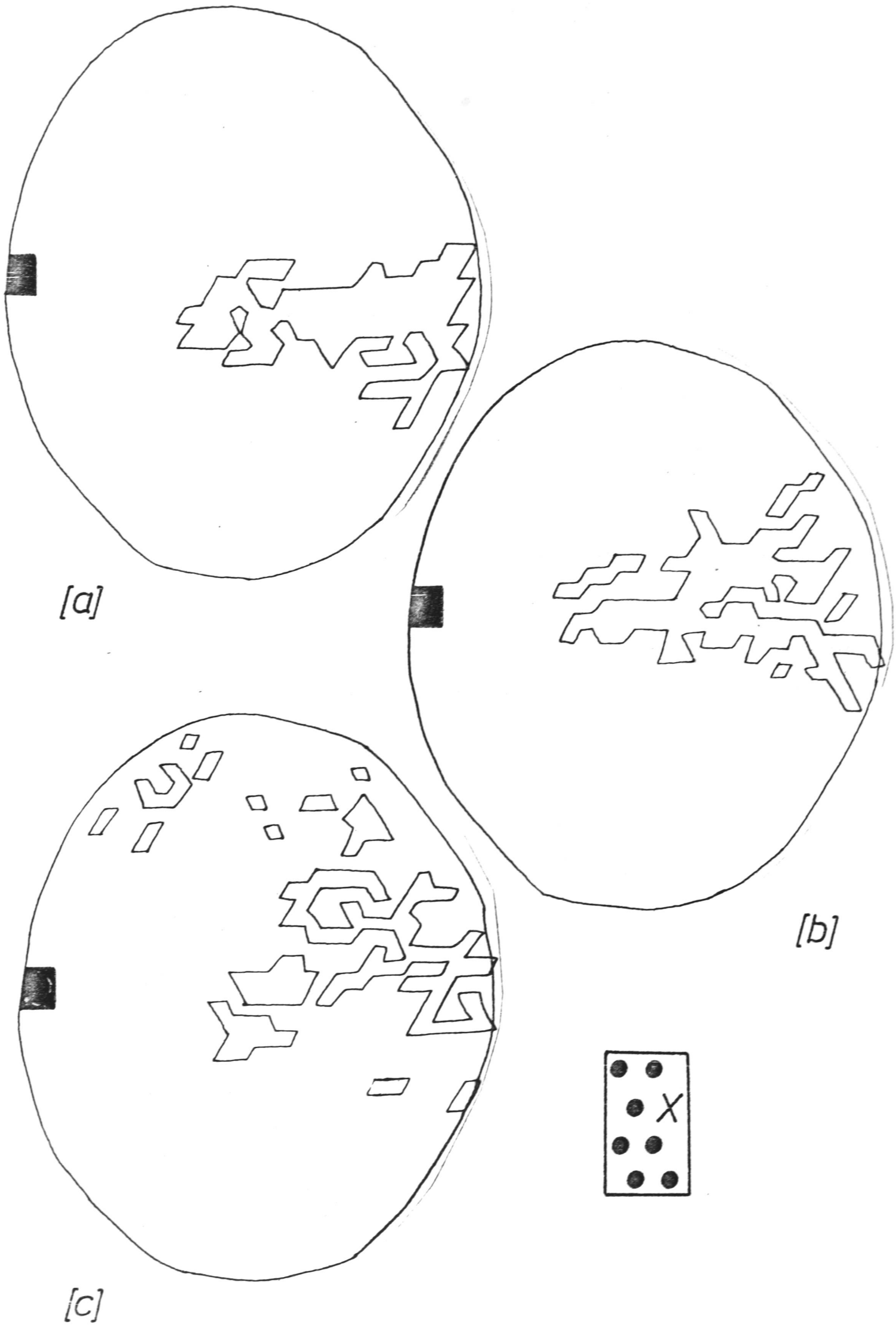
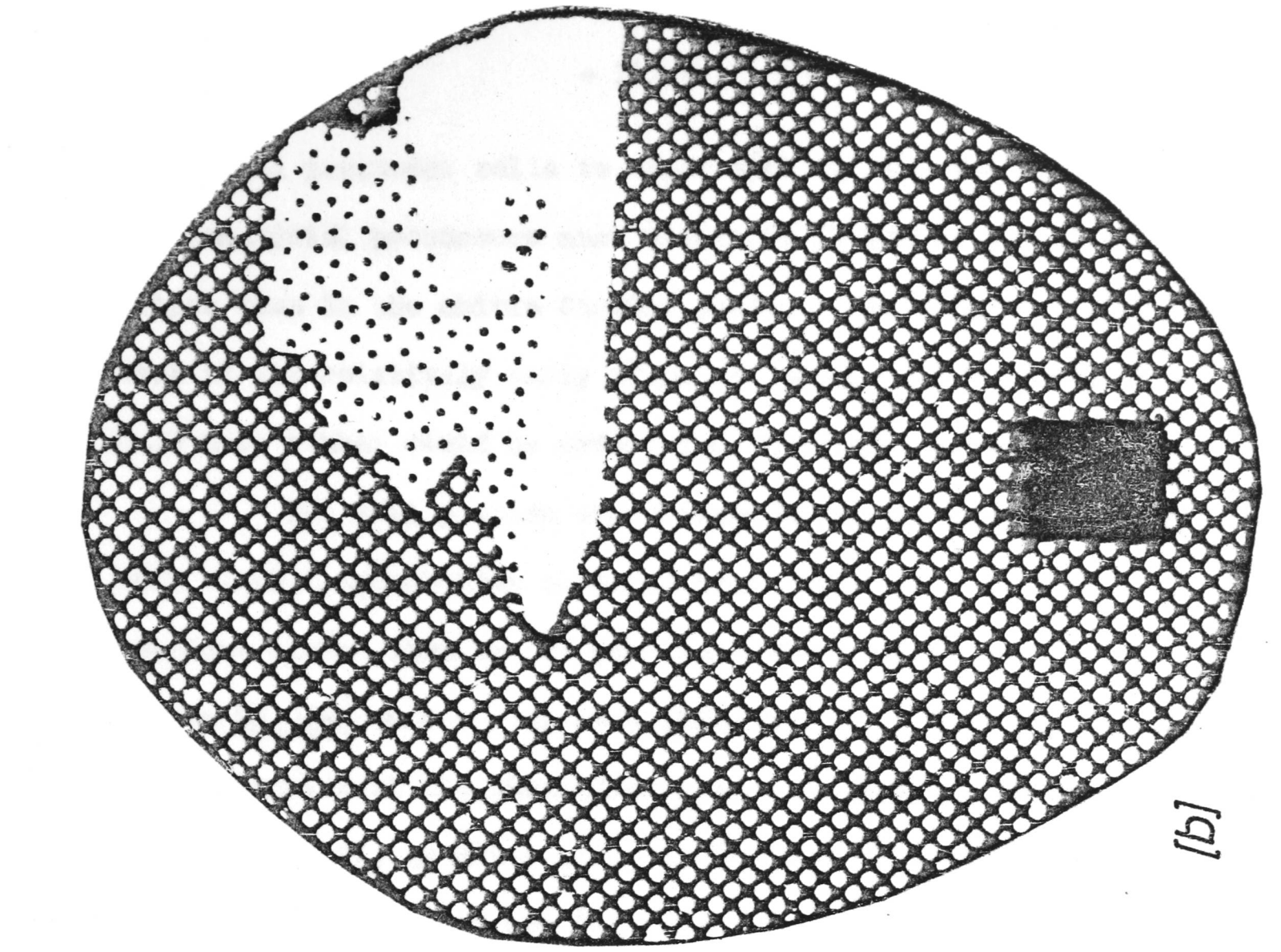


FIGURE 5.3 Examples of single clone patterns produced from 8 cell starter primordia, arranged as in inset box. The marked clone has developed from the cell marked 'X'. Note difference between 'smooth' clone shape of (a) and 'disperse' character of (c).



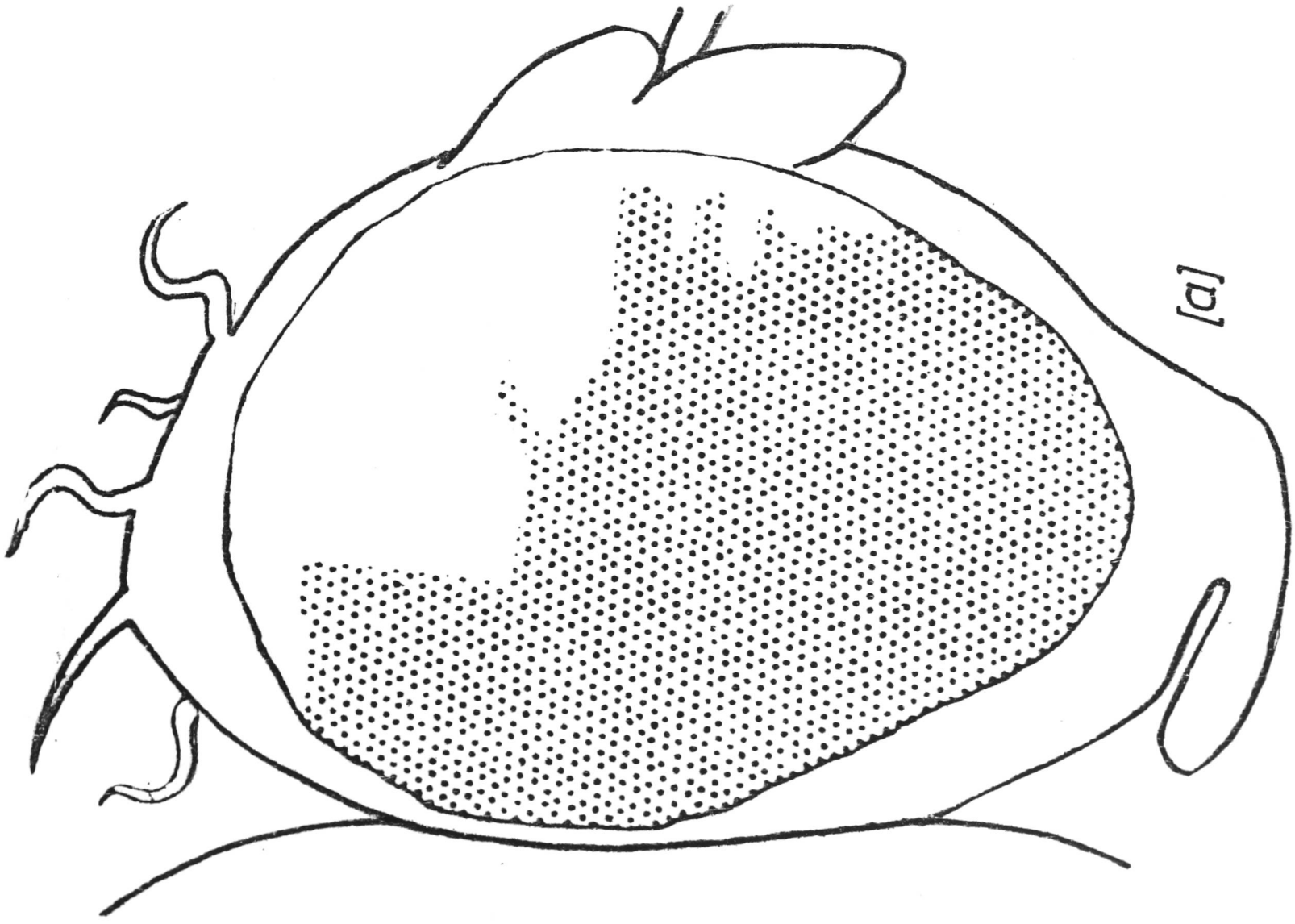


FIGURE 5.4 Two examples of observed clone patterns in the adult eye. (a) From Becker (1957). This clone includes a bristle marker, and the upper clone can be seen to extend onto the head cuticle. (b) From Baker (1967).

ommatidial precursor cells to start with or else the division in the ommatidial precursors must either be faster or go on for longer than in the chitin forming cells. The data does suggest that at the relatively early stage of 730 cells, a minimum of half this number would be determined ommatidial precursors.

The main problem encountered using the model was that much clone fragmentation occurred (see figure 5.3) and the coherent clones seen in the published pictures of eye pigment clones in vivo were rarely seen (figure 5.4). This was due to the properties of the simplified space clearing algorithm described above, by which cells on the edge of clones between the site to be cleared and the free edge of the cell mass could be pushed away from their parent clones. Several program variations were tried to limit this effect, including preferential cell 'stickyness' between cells in a clone, and using cells made up of more than one array position to try to buffer the pushing movement between parts of cells rather than always needing movement of a whole cell (Chap 3 p 80). These variations often gave rise to spurious results and complications, for example, if cells are made 'sticky' to their immediate neighbours, it may mean that a cell to divide cannot push freely in all directions: this may upset the random division patterns.

Since it is reasonably certain that the fragmentation observed in any one run is an artefact of the space clearing algorithm, it was decided to resort to an averaging technique to enable unfragmented clone patterns to be seen. To do this, ten runs of each simulation were carried out using different random

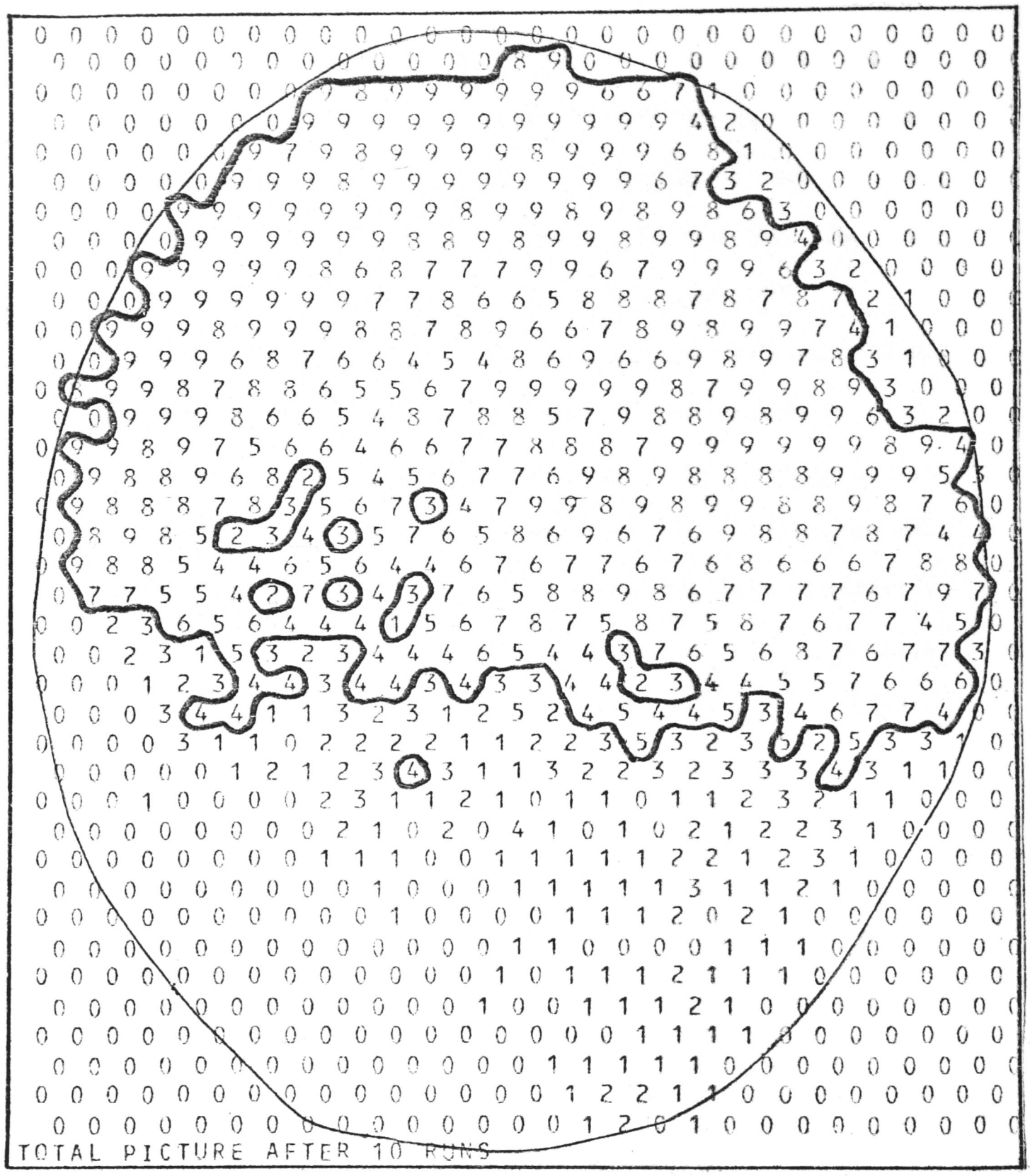


FIGURE 5.5 Composite 10 run picture. Figures refer to number of times out of ten that each array position appeared in the chosen clone. (For ease of display, '10' appearances is counted as '9').

starters each time. A composite picture was printed out at the end of the series of runs, giving a distribution diagram of the number of times each array position was part of the marked clone, (figure 5.5.) It was necessary to decide how many markings were sufficient to enable an array position to be scored as part of a composite clone: to do this the single run average size of each clone, determined from 10 single runs, was matched to the number of markings giving a composite clone of nearest size. In nearly all cases, this was 4 or more. When 16 cell primordia were used the figure had to be lowered to 3, as the small clones were more susceptible to separation.

RESULTS.

There were four main parameters to vary in running the model. These were the 'ellipse tightness' and growth pattern, the number of progenitor cells, and the identity of the marked cell within this number.

Ellipse tightness (the percentage area filled by cells before growing a set proportion) was set at 5% increase in area when 95% filled for all the results recorded here. Many other ratios were tried, but little observable effect, other than amplified clone fragmentations were observed with 'looser' membranes. The chosen ratio represents, therefore, the parameter setting giving minimum clone fragmentation for maximum speed - 'tighter' ellipse settings slowed the program down considerably, with no appreciable lessening of the fragmentation. Three ellipse growth patterns were used. Side (abbreviated to 'S'), with growth totally to one side, centre (C)

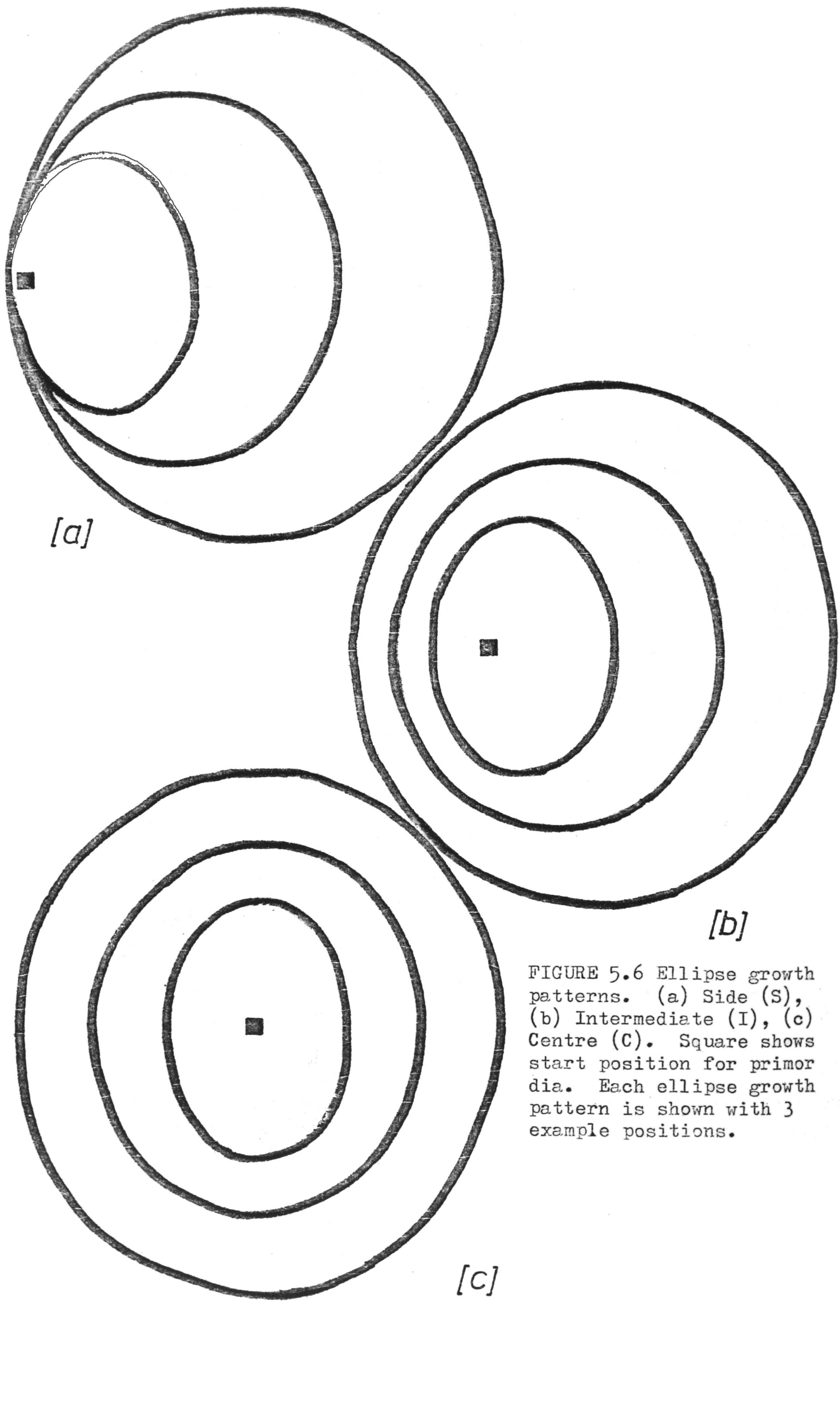


FIGURE 5.6 Ellipse growth patterns. (a) Side (S), (b) Intermediate (I), (c) Centre (C). Square shows start position for primor dia. Each ellipse growth pattern is shown with 3 example positions.

with growth radiating outwards from a point, and an intermediate (I) pattern. These growth patterns are shown in figure 5.6.

2, 4, 8 and 16 cell starting primordia were simulated. All possible marker cell positions in these primordia were run in the S pattern. Becker's observations (1957) suggest that pushing the growing clones from the posterior would give the closest clone patterns to those seen in vivo, and the S results were used as a yardstick with which to compare the results obtained from the C and I growth patterns. The results obtained in each case consisted of ten individual runs and a ten run composite picture.

The computer printout was used in four ways. The growth pattern of the marked clone in each individual run, the final shape and size of each clone and the composite picture were analysed; the 'twin spots' made by looking at the composite pictures of clones produced by adjacent primordial marker cells were also examined. The growth of each clone could only be observed in those cases where printouts of the clone in various stages of growth were obtained. As this would have resulted in too much printout to analyse, not all runs were treated in this manner. A version of the computer program was written which ran on a PDP-15 computer with cathode ray tube output, enabling the operator to watch the whole developmental process. Figure 5.7 shows the progressive growth of a clone derived by marking one cell of an 8 cell starting primordium.

(1) Average clone patterns produced by S growth pattern.

2 cell starters.

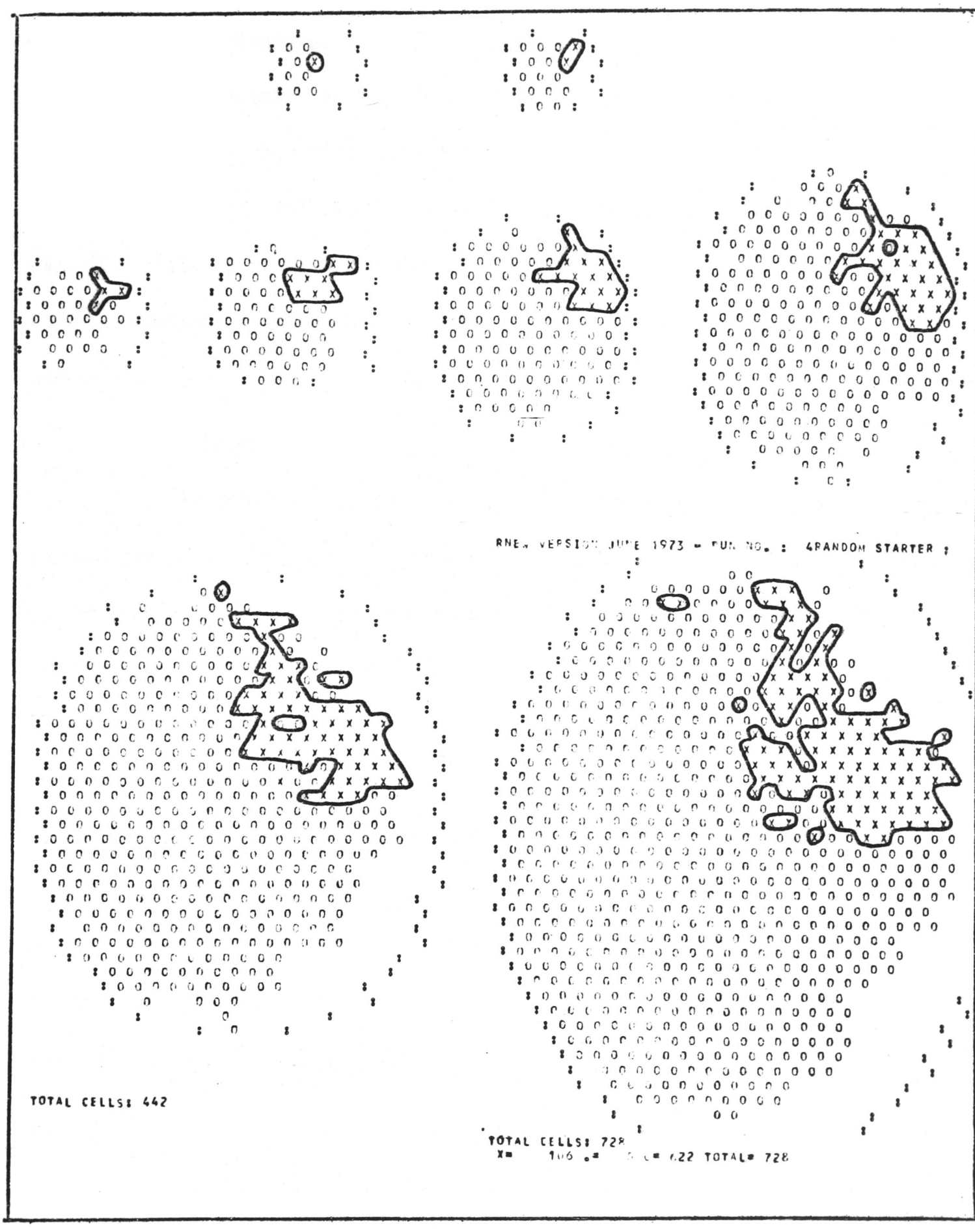


FIGURE 5.7 Clone growth for an individual 8 cell starting primordium with S growth pattern. Marking (:) shows boundary of ellipse. (X) is marked clone. (O) marks all other cells.

The final clone filled half of the available area with a roughly straight line separating marked clone from unmarked clone. The orientation of the dividing line varied with the relative orientation of the starter cells. If the two cells were placed with one directly anterior to the other, clones like that of figure 5.8a were produced. (All clone outlines have been directly traced from the computer printout and have been photographically reduced). If the cells were placed with one above the other, the clone shape of figure 5.8b was seen.

4 cell starters.

The clone shapes here differed depending upon the positional relationship of growing clone to the ellipse and to the surrounding cells not in the marked clone. Figure 5.9 shows the starting configuration of the 4 cells and the positions of the final clones, with all clones growing from left to right. The two rearmost clones are swept round with the growing ellipse, whilst the frontmost clone (marked 4) is pushed across the growth area to form a triangular section stretching from the centre to the front edge. The centre clone (3) formed a vertical band across the centre of the 'disc' - as this clone has been subject to most disruption from 'pushings', it has broken up more than the other three clones.

8 cell starters.

In these clones (figure 5.10), a dichotomy appears between clones which stretch across the growth area (clones 3, 4, 5 and 6) and the remainder of the clones, which sweep round the edge of the ellipse.

FIGURE 5.8, FIGURE 5.9, FIGURE 5.10, show 10 run average clone patterns with side (S) growth for 2, 4, and 8 cell primordia respectively. The inset figures show the positions in the starting primordia of the cells which eventually formed the numbered marked clones. The starting primordia positions are shown superimposed on the final clone pictures as black shapes.

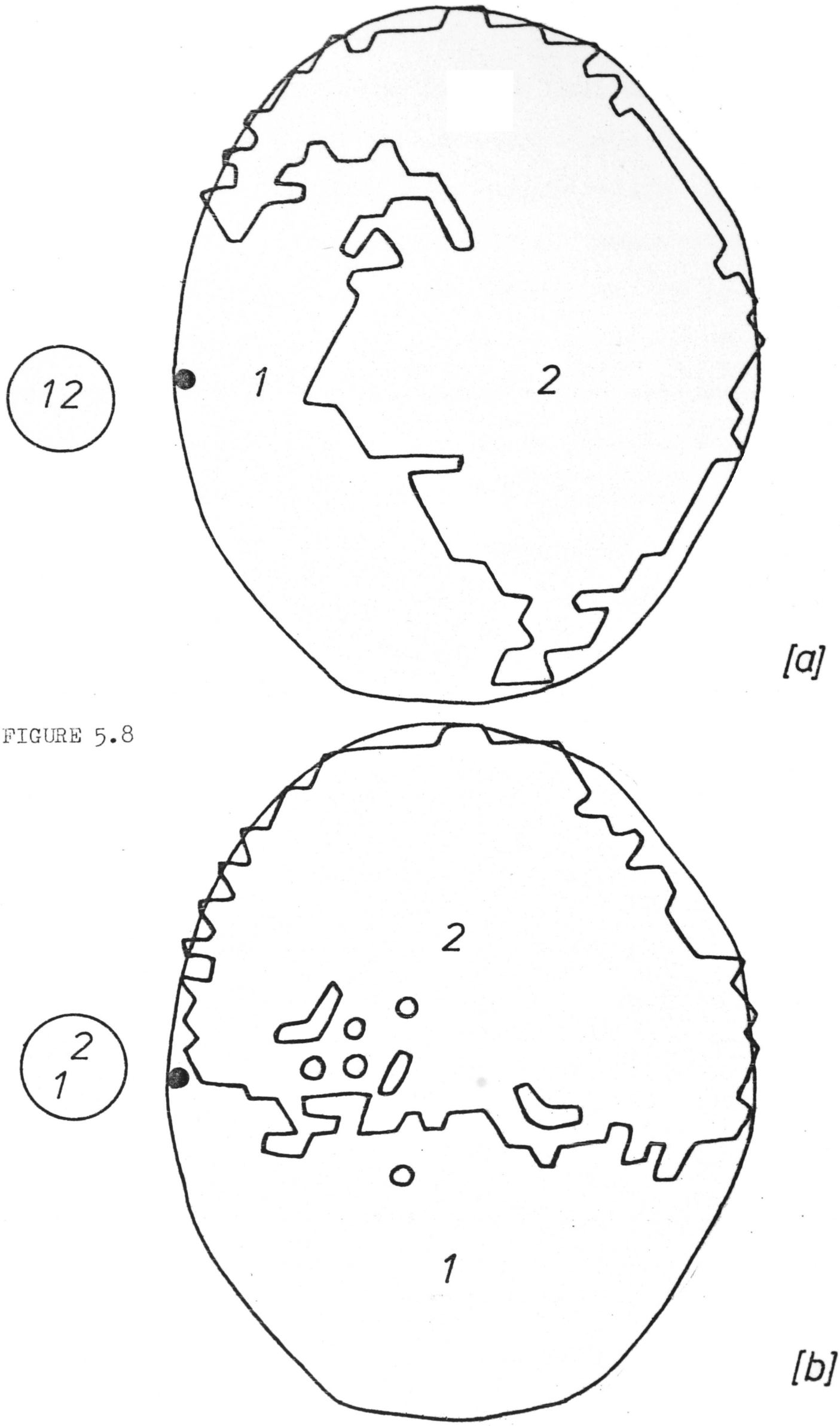


FIGURE 5.8

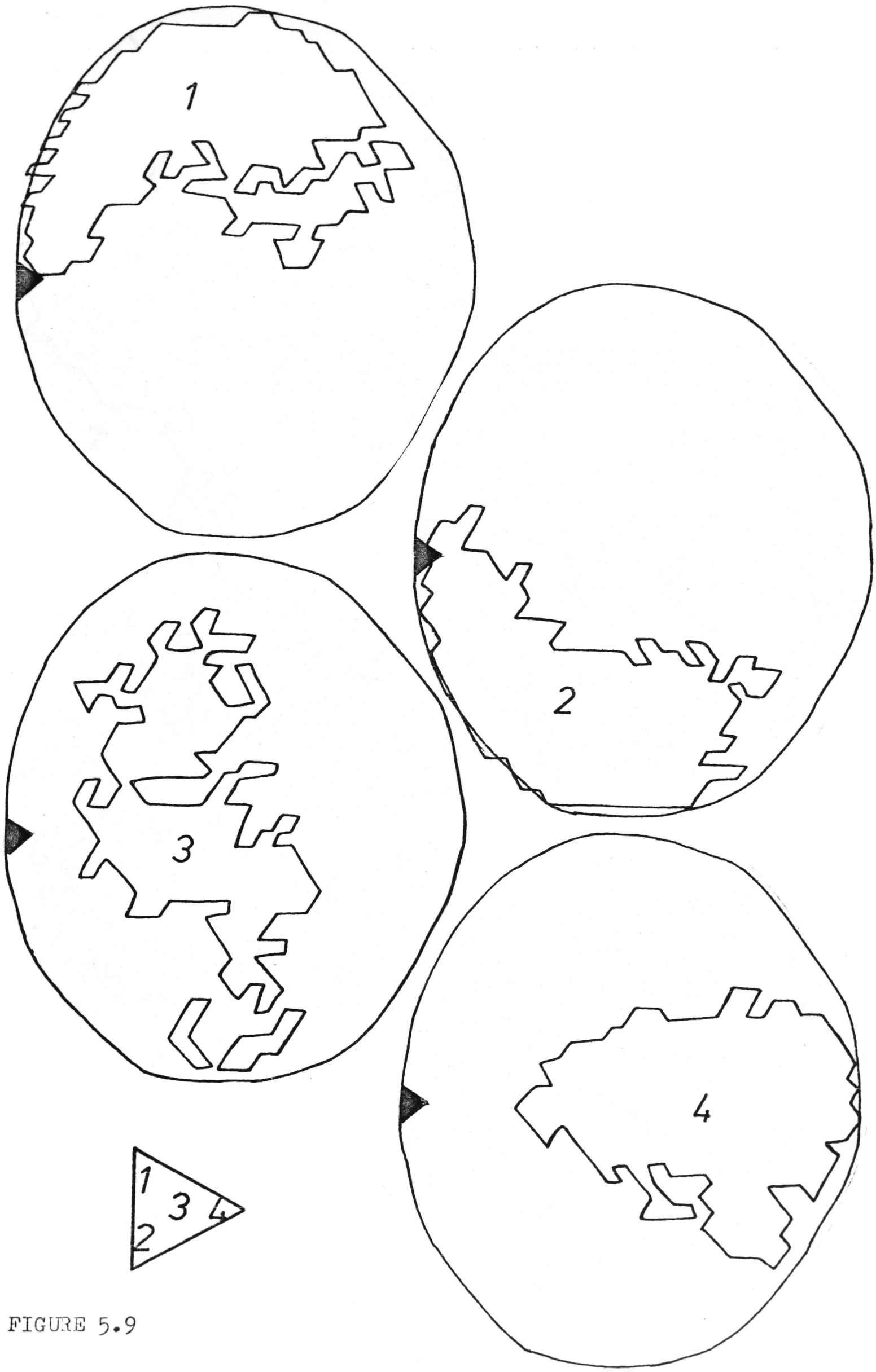


FIGURE 5.9

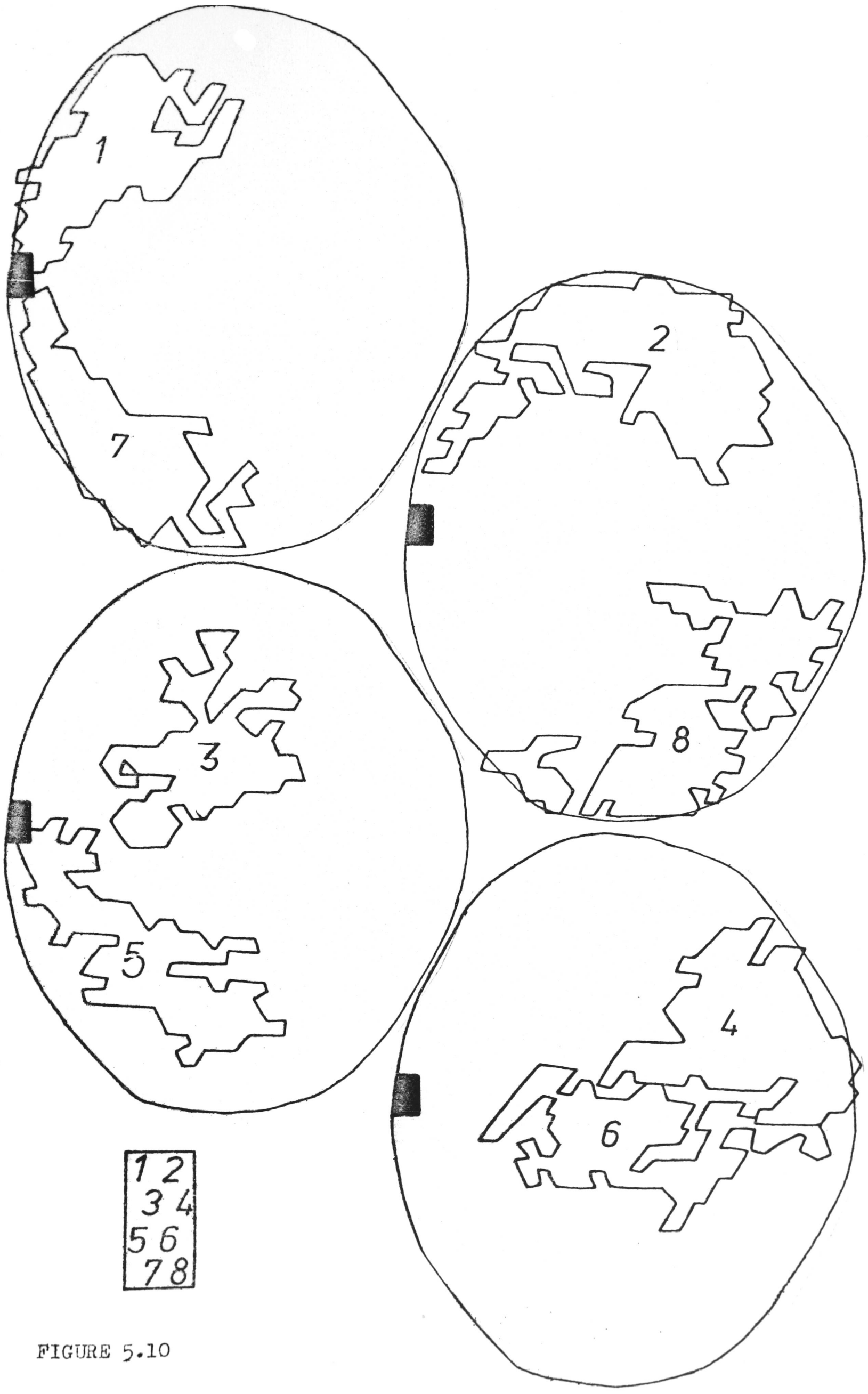
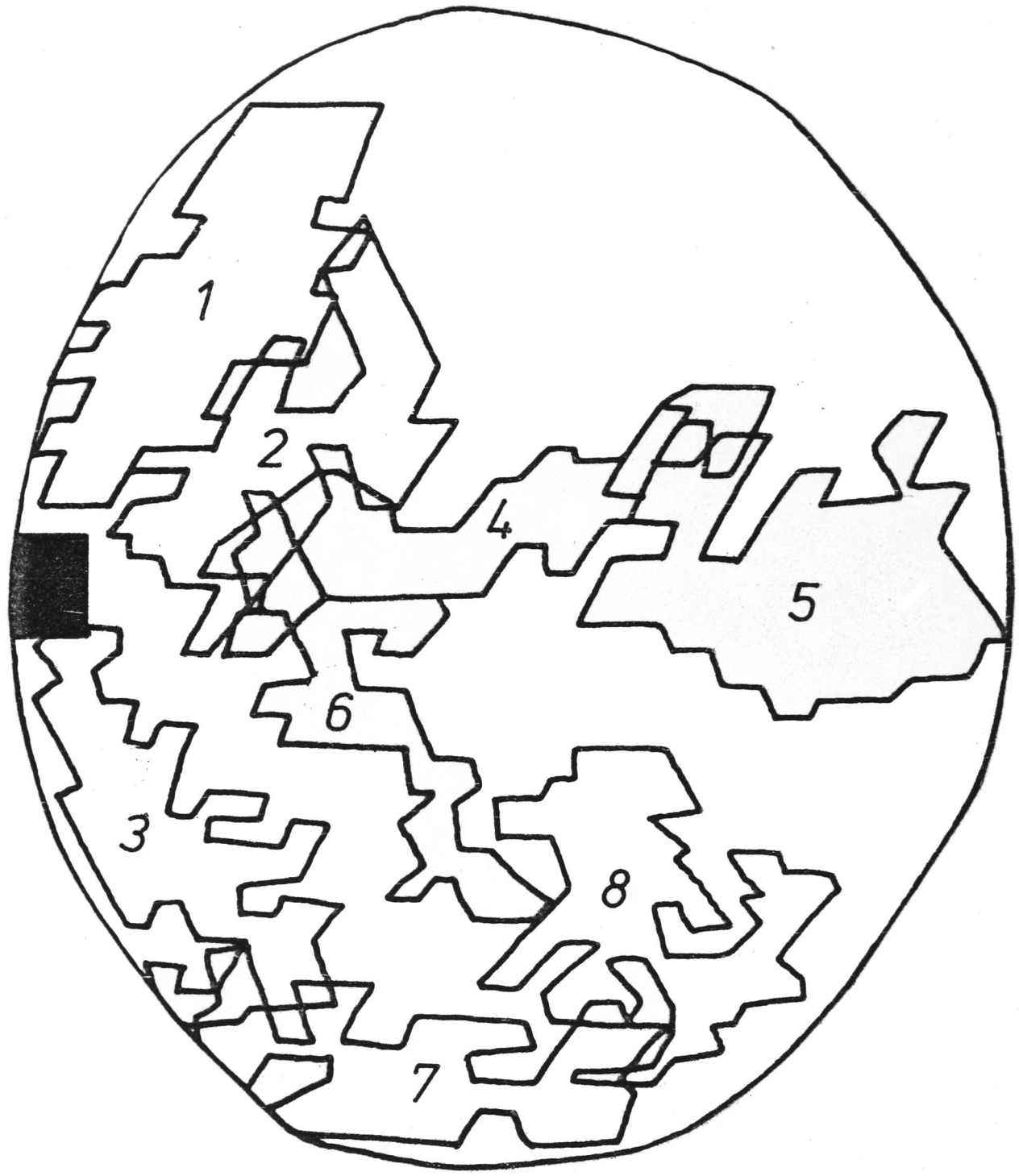


FIGURE 5.10



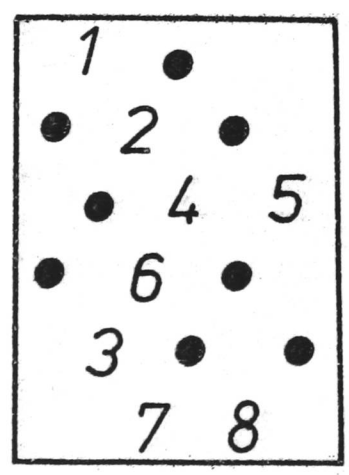


FIGURE 5.11 10 run average clone patterns as in previous three figures, this time for a 16 cell starting primordium. Only half of the clones are shown for clarity.

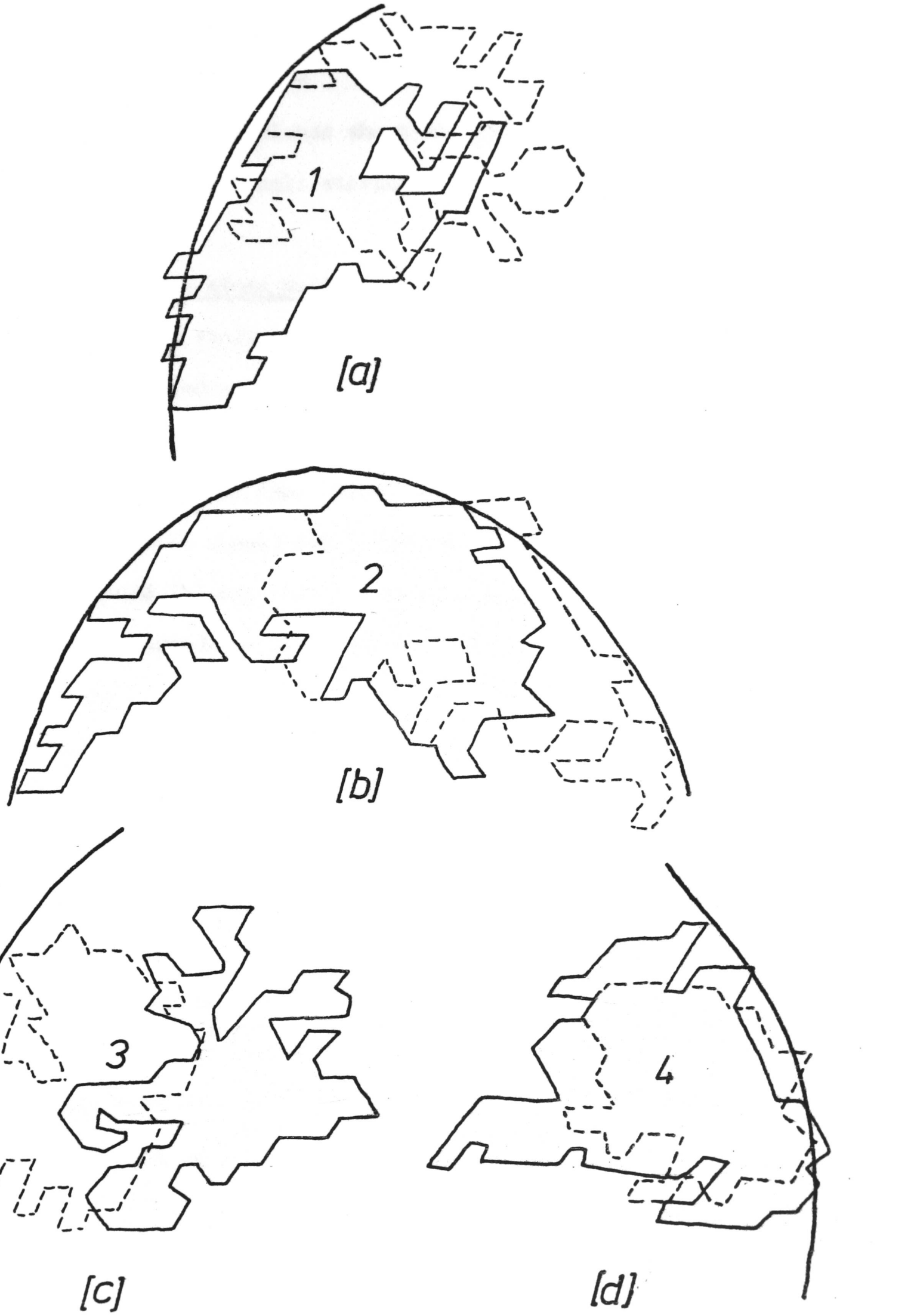


FIGURE 5.12 Average 10 run clone patterns - side (S) and central (C) growth compared. Dotted lines show C, solid lines show S . growth for the 4 upper cells of an 8 cell primordium. Solid curved lines represent edge of ellipse.

16 cell starters.

The 16 cell clones shown in figure 5.11 continue the trend seen with the 8 cell starters.

(2) Average clone patterns produced by I and C growth.

8 cell starters.

The tendency for clones to sweep around the edge of the growing ellipse was lost by changing the growth direction. Figure 5.12 shows the four upper clones formed from 8 initial cells for both S (solid lines) and C (dotted lines) growth patterns. Clones (a) and (b) especially demonstrate this. The I pattern showed an intermediate clone shape.

(3) Individual patterns.

As described above, the shape of the individual clones was affected by the pushing rule, and the coherence of the clones differed markedly depending on the position of the progenitor cell in the primordium. As was seen with the 4 cell starter S pattern, clones formed in the centre of the growth area were more susceptible to this type of separation. Figure 5.3 showed a comparison between coherent, non-coherent and 'average' final clones for an 8 cell starter S pattern.

(4) Clone growth patterns and relative clone sizes.

Because the computer program did not allow a cell to divide if all six division directions were unavailable, clones at the rear of the growth area in S growth patterns were slightly

smaller than those at the front, freely growing, edge. The maximum difference seen was about 10% of the total cell number of a clone. C patterns did not show this effect, as growth occurred equally in all directions.

(5) Twin spots.

Becker's original ideas (1957) concerning the change in orientation of cell divisions at the end of the first larval instar were based on the relative shapes of the juxtaposed twin spot clones. The ten run average computer clone shapes from each primordium were therefore compared, each with its neighbours, to see if any information on the relative orientation of pairs of clones derived from two adjacent cells could be obtained.

With the 8 cell starters, the clones which correspond to the central (ommatidial) region of the disc are, as Becker predicts, orientated side by side. (Compare, for example, figure 5.10 clones 4 and 6, 5 and 6). In the 16 cell primordia, some clone pairs (figure 5.11: 4 and 5, 6 and 8) show a tendency to align into anterior and posterior clones, as Becker's work suggests.

In brief, the above results indicate that constraints at the posterior of the growing computer 'cell' mass give clones orientated in a similar manner to those seen in the experimental studies.

DISCUSSION.

(a) A comparison with the experimental studies.

How far are the clone shapes found in this work duplicated in the eye pigment clones produced by position-effect variegation and somatic crossing over? The main problem in trying to draw direct parallels between simulation and *in vivo* clones is that apart from several isolated exceptions (Becker, 1957, p. 351) no clones extend onto the head cuticle (Becker used mutants with a bristle marker). The results obtained in this chapter represent the entire (half) head region, which includes head chitin as well as the eye. The clone distributions, therefore, represent more than Becker's clone distribution diagram (shown in figure 5.1c). We estimate that a large section of the centre of the computer printout will correspond to the 'ommatidial region' of the disc considered by Becker (1957). This estimate is based on the relative proportion of eye to head chitin shown in the fate map of the mature disc (Ouweneel, 1970).

If the results of sections (1) to (3) are compared with Becker and Baker's clones (Becker, 1957; 1966; Baker, 1967) it is clear that the posterior to anterior stretching of the clones so prevalent in the results of these authors is only duplicated by the S growth pattern. This suggests that there is an equivalent directional constraint in the eye disc. The existence of such directional constraints *in vivo* may mean that the clonal spreading around the edge of the growing cell mass is confined mainly to the chitinous tissue surrounding the ommatidial region. The possible importance of directional constraints as a

general morphogenetic mechanism will be discussed further below. Taking the centre area of the disc only, most of the clones which 'sweep' around the edge of the growing constraint are chitinous and, therefore, will not be seen as eye pigment clones. The total pattern of clone orientations closely ressembles that seen in the fly.

Becker suggests that the change in direction of the twin spot orintations is produced by a stimulus such as the meeting of brain and head disc at the end of the first larval instar. The results reported here indicate that the importance of this union might be simply to reinforce the constraining activity of the membrane already present around the disc. There are several different ways in which such a mechanism might work - either the division patterns before linking are completely random, or they are already constrained. As there are so few cells present in the eye portion of the disc at the end of the first larval instar (Becker estimates about 20) it will be very difficult to find out which is the case. The large size of the clones marked in the first larval instar may, in living system as well as in computer model, be 'too large' to be aligned with one posterior to the other, (i.e. the rearmost clone will always tend to push itself alongside the anterior one). This would suggest that the orientation change seen by Becker is an artefact of growth, and does not have any importance per se in disc development. Several of Becker's published clone drawings (Becker, 1966 figure 2, i and k) show clones produced in the second larval instar whose front edges deviate from the expected anterior/posterior pattern.

(b) Experimental verification of the 'constraint model'.

Although the results of the simulations suggest that early morphogenesis in the eye disc may be at least partly controlled by external constraints on cell divisions, first-hand morphological evidence is lacking. There is no easy way of tracking the division patterns of the presumptive ommatidial precursor cells in the larval disc, as the observation of clones depends on the production of pigment long after the events in the disc have occurred (pigment is not produced until the pupal period). It may be possible to use some enzyme marker which does not depend on differentiation in order to unmask its activity -β-glucuronidase is used as a marker in the study of clones in mammalian chimaeras

and then stain prepared mounts of larval discs to observe clone forming activity. Application of this method would first require isolation of a mutant suitable for somatic crossing over to be carried out with it.

As late as the third larval instar, when the ommatidial precursors are soon to begin differentiation, it is difficult to sort out which cells are involved in the formation of the ommatidial region as opposed to head chitin or bristle production. Sections have been cut off earlier discs (Steinberg, 1941) but the absence of characteristic features in the presumptive ommatidial cells prior to the time of 'clustering' at the end of the third larval period makes interpretation difficult. Ouweneel's fate map of the eye disc (Ouweneel, 1970), constructed from fragmentation and transplantation studies, shows that a fine determination exists at the end of the third larval instar.

If the brain to eye-disc connection is important in setting the orientation of cell divisions in the eye disc, then removal of the link may throw light on the processes at work. This experiment would have no guarantee of success as the early stages of development may require interaction between them. Trujillo-Cenoz and Melamed (1973) have noted that axonal connections are present between eye-disc and brain in the earliest larval stages in the blow-fly, and that these fibres originate in the brain. It is known that the main nerve connections develop from eye to brain, probably during the ommatidial formation stage (Power, 1943). Power has shown for example that the external optic glomerulus also forms from the eye. The early axonal connections may, therefore, indicate an active relationship between eye and brain in the early larval stages.

Disc culture in vitro, a potentially important technique especially since the recent introduction of defined insect media, has only been carried out in the third larval instar (Kuroda and Yamaguchi, 1955). However, both these experiments and those of Goldsmith using transplanted third instar discs (Goldsmith, 1968) show that axonal connections are not necessary for differentiation, at that stage.

(c) The nature of the constraint mechanism.

A physical basis for the contraints proposed in this thesis might be provided by the peripodial membrane and non-cellular basement lamina which surround the disc. Limited growth in these layers constraining the underlying disc tissue, together with the larval brain to eye-disc link which may provide a solid

'back-plate' against which the larval disc cells cannot divide, could push the cells into dividing forwards.

The growth pattern that would result if such constraints are acting is seen in the mutant lethal 2 giant discs (Bryant and Schubiger, 1971). The imaginal discs of this mutant exhibit increased growth - the antennal disc and front surface of the eye disc are overgrown and distorted in the mutant, whilst the rear eye disc surface has the same flat appearance in both mutant and wild type discs. The extra growth activity of the mutant may be actively constrained from pushing backwards.

There are two phenomena in eye disc development that have not been taken into account in the computer model. The size of the posterior clone of Becker's twin spots induced in the second larval instar was found to be consistently less than the size of the anterior clone spot. This suggests that there are less cell divisions in the rear part of the disc during the time of clone formation, and a representation of this was not included in the model. The larval disc is also convoluted - the single layered epithelium buckles and bends - this may also add complications to the simple two dimensional model described here.

It may be possible to find out the orientation of cell divisions on the head cuticle by somatic crossing over experiments with cuticle markers. If, as would be expected from these simulations, there is 'spreading' around the edge in the regions closest to the membrane in the disc, this would further suggest the existence of the postulated constraint mechanism.

(d) Constraints and compartmentalization in the head disc.

Garcia-Bellido, Ripoll and Morata (1973) have defined compartmentalization as a "developmental restriction between disc regions". Cells determined to be a part of one 'compartment' cannot overgrow into another 'compartment', and so a "splitting mechanism segregating different groups of cells from a previously homogeneous and contiguous cell population exists". Garcia-Bellido has demonstrated the phenomenon of compartmentation in the wing disc of Drosophila (Garcia-Bellido et al., 1973). In a similar vein, Lawrence (1973), analysing somatic crossing over induced clones on the abdomen of Oncopeltus, has shown that the later the clones are marked the smaller their size. After a critical time, they do not cross 'intersegmental boundaries', when they occupy a single 'segment quadrant' (dorsal or ventral, left or right).

In the eye disc, the clones produced by both somatic crossing over techniques appear integral, and only rarely show splitting into small, separate clumps of tagged cells. The same is true for antennal clones (Postlethwaite and Schneiderman, 1971), leg (Tokunaga, 1962) and wing disc (Bryant, 1967). In Lawrence's studies with Oncopeltus this does not seem to be the case, and large clones may split into as many as nine smaller clones (see Lawrence, 1973, p. 687). One of the basic requirements of a compartmentalization mechanism is clonal integrality - if the cells in a newly formed compartment are allowed to separate and mingle with their neighbours, the compartmental boundaries will soon be broken down. The clonal integrality of insect imaginal discs has

hitherto been assumed to be a property of the single-layered epidermal structure of the discs. However, the Oncopeltus abdomen also develops from a single layer of cells, but apart from the orientation of cells at the anterior margin of each abdominal segment (Lawrence, 1973) the mitoses are 'randomly oriented, and the segment grows evenly over all'. Lawrence suggests, in fact, that the amount of cell division is under a general probabilistic control.

It may be that in the Oncopeltus abdomen, natural borders are set down early (the presumptive epidermis becomes effectively segmented during blastoderm formation), and all the cells are within preset compartments. Because of the borders, it is not necessary for the cells to have their division directions controlled. The mixing which does occur in Oncopeltus is slight, and does not deviate significantly from the theory that little cell migration takes place in disc development. The clones on the Oncopeltus abdomen might, in fact, be taken as the norm for clonal growth in two dimensions when there are no constraining factors.

In the Drosophila discs, a different situation exists, Here, early borders may not be physically set down between different groups of determined cells, and the cells may have to 'decide for themselves' how the compartmentation is to be carried out. I have indicated in this chapter how the natural separation of cells destined to form ommatidial and chitinous parts of the head may occur. If the primordia of the cuticular region are initially placed behind the ommatidial precursor cells, they may naturally sweep round the edge of the growing disc. The

concentric nature of Drosophila discs is well known, and Ouweneel (1973) has put forward the observation that the most external parts of the adult structures formed by each disc tends to be derived from the central parts of the disc. It may be that in discs structurally more complex than the eye (for example, the wing, where a concentric, ring like arrangement is seen), the sweeping process is carried out in the interior of the disc and not just around the membrane.

(e) Constraints as a general morphogenetic mechanism.

The directed division orientations of the eye are also seen in other Drosophila imaginal structures. Tokunaga (1962) has analysed cell clone lineage on the male foreleg - the clones here tend to extend longitudinally down the leg. The same pattern has been observed in the leg of the honey bee Apis mellifera (Mehling, 1915). The clones extending laterally across the wing of Drosophila (Bryant, 1967) have also been seen on the wings of the moth Colias (Hovanitz, 1965). Nothiger and Dubendorfer (1971) have found similarly shaped clones to those seen in the Drosophila eye in the eye of the house-fly Musca domestica after mitotic recombination.

The occurrence of such non-random cell divisions in insect imaginal discs, therefore, suggests that there may be some general mechanism(s) in operation, and I have indicated in the previous section how this might be linked to the current ideas on 'compartmentation'. There may also be similar mechanisms at work outside the insect world. A good example of close interaction between tissue and overlying membrane is seen in the vertebrate

limb bud, where the apical ectodermal ridge has been shown to be essential for normal development. It is the underlying mesoderm that forms the limb proper, but removal of the ectoderm has a catastrophic effect on limb development (Janners and Searls, 1971). These authors suggest that the ridge might be an outgrowth inducer that 'permits growth in the proper direction and thereby shapes the growing mesoblast'. If the constraints of the sort modelled in this thesis act on the limb bud, then the importance of the ectodermal ridge might involve 'paying out' ectodermal covering to maintain the correct division pattern in the mesoderm. A computer model putting forward such a controlling function for the ectoderm has been described by Mitolo (1973).

CHAPTER SIX

DIFFERENTIATION IN THE DROSOPHILA EYE DISC

INTRODUCTION.

We have discussed the early morphogenesis of the Drosophila eye disc, and how pigment clones can be used as markers to follow this development. In the present section we will look at another aspect of eye disc development; how the differentiation signal 'switching on' the determined ommatidial region operates. Knowing how this works may be of general importance in better understanding the activation of determined cells in insect imaginal discs.

There is much evidence that the imaginal disc cells of Drosophila are determined early in their development. This has been shown by the many transplantation experiments carried out on various discs (e.g. Bodenstein, 1939). However, a signal is necessary before differentiation can occur. The nature of this signal is roughly known. It is a stimulus produced by or associated with the change from larva to pupa at the end of the third larval instar, itself produced by a high level of the moulting hormone, ecdysone, together with a low level of juvenile hormone. (Gilbert and Schneiderman, 1960; Oberlander, 1969). In adult flies, which have no detectable ecdysone (Shaaya and Karlson, 1965), transplanted disc cells can multiply indefinitely, and only differentiate when transplanted into larval hosts which are allowed to undergo metamorphosis.

Disc cells at all stages of larval development cannot

react to the metamorphosis 'switch': Bodenstein (1939) showed that 50 hour* eye discs give incomplete differentiation if transplanted into larvae about to metamorphose. It seems that there is a period at around the beginning of the third larval instar (72 hours) at which competence is reached: this has been found by Bodenstein (loc. cit.) and Mindek (1972). Schlapter (1963) has shown that if in vitro cultured eye discs are given low hormone levels, eye pigments only are formed.

Mindek has also shown that the competence of a disc may be very specific. If an early larval eye disc is transplanted to a larva about to pupate, the implant goes on growing right through the metamorphosis of the host. If the disc is then transplanted to a younger host, it will differentiate when it has reached the 'competent' age, and the host undergoes metamorphosis.

The differentiation signal is therefore time specific. Is there any information about the mechanism of initiation of the signal in particular discs? The best evidence comes from studies of mutants, especially those which express themselves first in the last larval instar. In Drosophila homozygous for lethal-2 giant larvae, the imaginal discs grow to an abnormal size, are disorganized, and are unable to begin metamorphosis - either the signal does not occur, or else it is 'misinterpreted'.

Note: *As in the last chapter, all ages, unless otherwise stated, are from the onset of larval development, 24 hours after hatching.

In eye mutants like bar, lozenge, and kidney, there is limited normal differentiation, expressed by a lesser number of ommatidia seen in the adult. Factors like temperature shocks in the second larval instar can give an increased ommatidial number in bar, indicating that it may well be the signal to differentiate that is upset in this mutant.

Because bar possesses a dosing series, with further duplications of the gene giving lesser and lesser numbers of ommatidia placed towards the rear of the normal wild type ommatidial region, and also because the ommatidial numbers, and perimeter shapes of the ommatidial region are highly repeatable, unlike in other eye mutants like eyeless, a study of possible models of the mode of action of this mutant has been undertaken. The aims of this study were to produce model systems that included provision for both known and hypothesized important parameters in disc differentiation. These parameters could then be varied to see if the model could be made to duplicate the experimental observations of the actual bar series. A second mutant, eyeless Russian also has a repeatable but smaller, number of ommatidia than wild type, this time in the centre of the wild type determined ommatidial area. A bar series has been also made by Andrews (1970) on this background, and so the activity of two genes acting in concert could be studied.

As the bar dosing series is so repeatable, we thus have a very potent tool for studying differentiation in the eye disc. If we can produce a set of parameters which, when varied in a simple manner give the ommatidial reductions, and eye

perimeter shapes seen in the bar mutant, it may be possible to throw light on the mechanism of differentiation in the eye disc. Before discussing the strategy for such an approach, we will review what is known about eye disc development at the time differentiation is about to begin.

DIFFERENTIATION IN THE EYE DISC.

From the 'four cell stage' onwards (see last chapter for review of earlier disc development), cells that are to all extents and purposes morphologically undifferentiated divide and develop to give rise to a cell system of great complexity. Not only are the individual ommatidia created, but so also are interfacet setae (small hairs), and the chitinous border of the compound eye. The bristles, ocelli, and head chitin also develop. The ommatidia are placed symmetrically, in a hexagonal pattern. What are the underlying causal mechanisms at work?

There is much evidence to suggest that a 'differentiation stream' travels in a posterior to anterior direction over the insect eye disc during the third larval instar, and that it is this that starts cells grouping and dividing locally to form ommatidia. A lot of this evidence comes from species outside the diptera. Wolsky (1949) cauterised different parts of the eye disc of the moth Bombyx during pupation. He found that the pattern of ommatidia seen in the treated adults fitted well with the hypothesis that there is a posterior to anterior wave of differentiation. If cauterisation was started at the beginning of pupation, at the back of the eye disc, practically no ommatidia

were seen in the adult. If the same place was treated late in pupation, only the rear of the eye was affected. Cauterisation of the front of the eye early on gave only localized defects, while late cauterisation in the same place stopped differentiation at the front of the eye.

The differentiation process in Bombyx takes place some 24-28 hours after pupation, and is therefore later than in Drosophila. A similar result has been obtained by White (1961) with the mosquito Aedes aegypti. In the mosquito, ommatidial differentiation is easily followed because of the associated eye pigment formation. The pigment is observed to travel forward in a band increasing in width in the fourth larval instar (Aedes has one more larval moult than Drosophila). White has shown that if epidermal tissue from outside the presumptive ommatidial region is implanted in the path of the pigment band, the developmental pattern may be changed, the implant acting as a 'barrier' to further differentiation. White also observed mitotic activity spreading from posterior to anterior of the eye at this time.

Other evidence for the differentiation stream has come from studies of the Odonata (dragonflies). In this order, the number of ommatidia may vary from the basic number, 7, to 270. 7 ommatidia initially appear, and act as a 'budding zone' (Ando, 1957), from which the other numbers of ommatidia derive. In species with more than 7 ommatidia, the additional ommatidia are added at the anterior of the eye, and the original group are the posteriormost ommatidia. Seidel (1935) has also observed what he calls the "Differentiationsstrom" in Platycnemis. He found

that caudal irradiation of the eye anlage resulted in more extensive damage than irradiation of the other parts of the anlage. Bucklin (1952, quoted in White, 1961) has also observed this phenomenon in the grasshopper. Likewise, Umbach (1934) and Ludke (1940) have noted the presence of a differentiation stream in Ephestia and Notonecta respectively. Evidence suggests that Drosophila also fits in with these observations. Becker (1956) treated third instar larvae with X-rays, and observed irregularities in facet patterns. These irregularities varied in position depending on the time of administration of the radiation - a 'sensitive' vertical line of scarring migrated across the anlage from posterior to anterior. This scarring has also been observed by Poodry, Hall and Suzuki (1973). Fristrom (1972) investigated the eye disc of the bar mutant, finding that a wave of 'cell death' migrated across the eye from posterior to anterior at the beginning of the third larval instar. She suggested that the cells die, instead of differentiating into the component parts of ommatidia in this mutant, and that the changing positions of the cell death may be indicative of a differentiation wave.

If a wave of this kind is in operation, there are several ways in which differentiation might be initiated. The cells at the rear of the disc might be 'older' than those at the anterior, and therefore be ready to go through the next division earlier. If the cells have some sort of 'timing' mechanism in them, initiating differentiation after a certain number of cell divisions, the local assembly into ommatidia could start at the same time. Alternatively, some kind of active switching signal could travel

forward across the eye disc. In order to prove or disprove the latter theory, several papers have dealt with disc culture in vitro, to see if isolation prevents differentiation. Kuroda and Yamaguchi (1955) investigated the effect of culturing 71 hour larval discs with and without the attached 'cephalic complex' (brain and optic glomeruli); they recorded increased differentiation in the presence of the complex, although this could have been due to the lesser surgical shock of maintaining the brain link.

Wolsky and Huxley (1936) found that mutants of the crustacean Gammarus have degenerate eyes because of the lack of a determinative stimulus from the optic tract of the central nervous system.

Gottchewski and Querner (1962) later showed the spread of injected fluorochrome from the brain to optic disc in Drosophila larval explants. They found that there were two distinct parts of the brain with a separating tissue impervious to fluorochrome. If the latter substance was added to the anterior part (in mid-third larval explants of about 80 hrs. larval development), it was found that the fluorochrome travelled first to the eye, then the antennal disc, but not in the reverse direction. If the operation was repeated at a much earlier stage, (before 40 hrs.) essentially the same observations were seen, except that the fluorochromes could diffuse over to the opposite antennal/eye disc complex. They could still not get back to the brain tissue.

We have already seen that eye discs can develop when

placed elsewhere in the embryo, but do not differentiate in the adult. This suggests that some sort of external signal is required. El Shatoury and Waddington (1957) saw that cells from the larval lymph glands degenerate and release cells called oikocytes at the end of the second larval instar; these cells then migrate to the imaginal buds. At about 85 hours, those in the eye disc exhibit nuclear pycnosis and deposit melanin, later becoming vacuolarised and undergoing disintegration. El Shatoury and Waddington suggested that the lymph gland cells may play a part in differentiation.

The pattern of the ommatidial layout in the eye may also give some clues to the nature of the differentiation process. Franceschini and Kirschfeld (1971) have noted that the eye is in two symmetrical halves, dorsal and ventral. The 8 rhabdomeres at each ommatidium are arranged in a characteristic pattern and sectioning of the adult eye shows that those in each half are orientated as if they were mirror images. Two questions may be posed. How do the cell groups come to lie in the same relative orientations every time, and why are there two distinct orientations of rhabdomere groups? I would suggest that perhaps the local assumbly reaction of individual groups of cells into ommatidia is a 'self assembly' process brought about by the characteristics of the cells themselves, and the hexagonal alignment of the whole rhabdomere groups is a continuation of this process on a higher level. There is much evidence that this sort of local assumbly goes on at the subcellular level - in virus particles and collagen molecules for example.

The evidence is in favour, therefore, of some kind of differentiation wave, passing, in Drosophila, from posterior to anterior at about 70 hours of development. There are other factors which lead to a better understanding of the process, and these are shown by the previous studies on the development of bar.

THE BAR-EYE MUTANT.

Unlike other mutants which lessen the number of ommatidia in Drosophila, in an irregular manner, the bar phenotype has a characteristic narrow band of facets placed towards the rear of the normal outline of the wild-type eye. Bar is sex-linked and semi-dominant in the female, the $B/+^{B}$ heterozygote having fewer ommatidia than wild-type, but more than the B/B homozygote. A more extreme type of bar 'ultra bar' was found by Zeleny (1919). Sturtevant found that reversion to wild-type from B/B (with a frequency of about 1:1600) was always accompanied by crossing over. Sturtevant also suggested that unequal crossing-over between the bar loci was responsible for the reversion. The formation of wild-type and ultrabar from bar occurred when unequal crossing over resulted in two copies of the bar gene on one chromatid and none on the other. It was also seen that the heterozygous ultrabar ($BB/+^{B}$) female had fewer ommatidia than the homozygous bar (B/B).

Bridges (1936) cytologically verified the gene duplication theory, showing that bar itself is associated with a duplication of the region 16A1-A7 on the X - chromosome. Ultrabar has three such regions. Rapoport (1940) made 5-9 fold tandem repetitions of the bar region. With the data he obtained, he was

able to demonstrate a non-linear relation between number of bar regions on the X - chromosome, and ommatidial number. The section below on 'dosing data in bar' describes the arithmetic of this relationship.

Embryology of bar.

Chen first observed (1929) that the bar eye disc was smaller than the wild-type disc at the earliest observed stage (about 36 hours of larval development). His observations were confirmed by Steinberg (1941) who measured the area of both wild-type and bar discs at various stages during larval development. However, Steinberg considered disc size as a growth marker. By doing this he concluded that, unlike the wild-type disc, growth in the bar eye disc is not exponential. This conflicts with Chevais' (1943) observation that cell number increase is, in fact, exponential. The reason for this exponential increase not being 'expressed' by the disc may be that during disc growth, the disc epithelium is thrown up into large folds which could effectively mask any external volume increases.

The optic glomeruli are reduced in size in the bar mutant, showing a similarity with other eye mutants like eyeless. The head of the bar homo- or hemi- zygote also seems to be slightly reduced in the anterior, but this is only based on personal observation, and I have not found mention of it in the literature.

There is a critical period between about 40-60 hours of larval development when the ommatidial expression in the adult can be altered by temperature (Margolis, 1935; Hersch, 1930), or by adding certain chemicals (Kaji, 1960). The range of the

chemicals added includes ammonium lactate, which, when added at 60 hours of larval development, could induce eyes of apparently the normal shape, with the wild-type number of ommatidia; other successful substances are magnesium ions; and nucleotides.

Kuroda and Yamaguchi (1955) explanted 71 hour larval discs from the wild type Oregon strain, and bar. If the cephalic complexes were separated from each and swapped round, the Oregon eye disc tended to give bar ommatidial development and vice versa. These isolated findings, suggesting non-autonomy of bar eye development, are eclipsed by other work.

Sturtevant (1927) studied white eyed mosaics of bar individuals, and found that the mosaic patches were not restricted to the same extent as the bar background. Although in all cases the white patches were joined to the bar eyes, they distorted the normal bar eye shape, especially at the anterior. Sturtevant's work could be criticised on the grounds that his induced patches were male in a female background. As a slight difference in male and female response to the bar gene has been noted (the male B/Y has about 20% more ommatidia than the female B/B), a limited number of extra white ommatidia might be expected. DeMarinis (1952), however, repeated the mosaic experiments using a somatic crossing over technique, and he obtained large white female patches in a female bar background. In one case, the number of ommatidia in a +/B heterozygote was increased almost to the wild type number. This, together with Steinberg's (1941) observation that bar eye discs transplanted after 24 hours larval development into wild-type host larvae develop bar eyes, rather

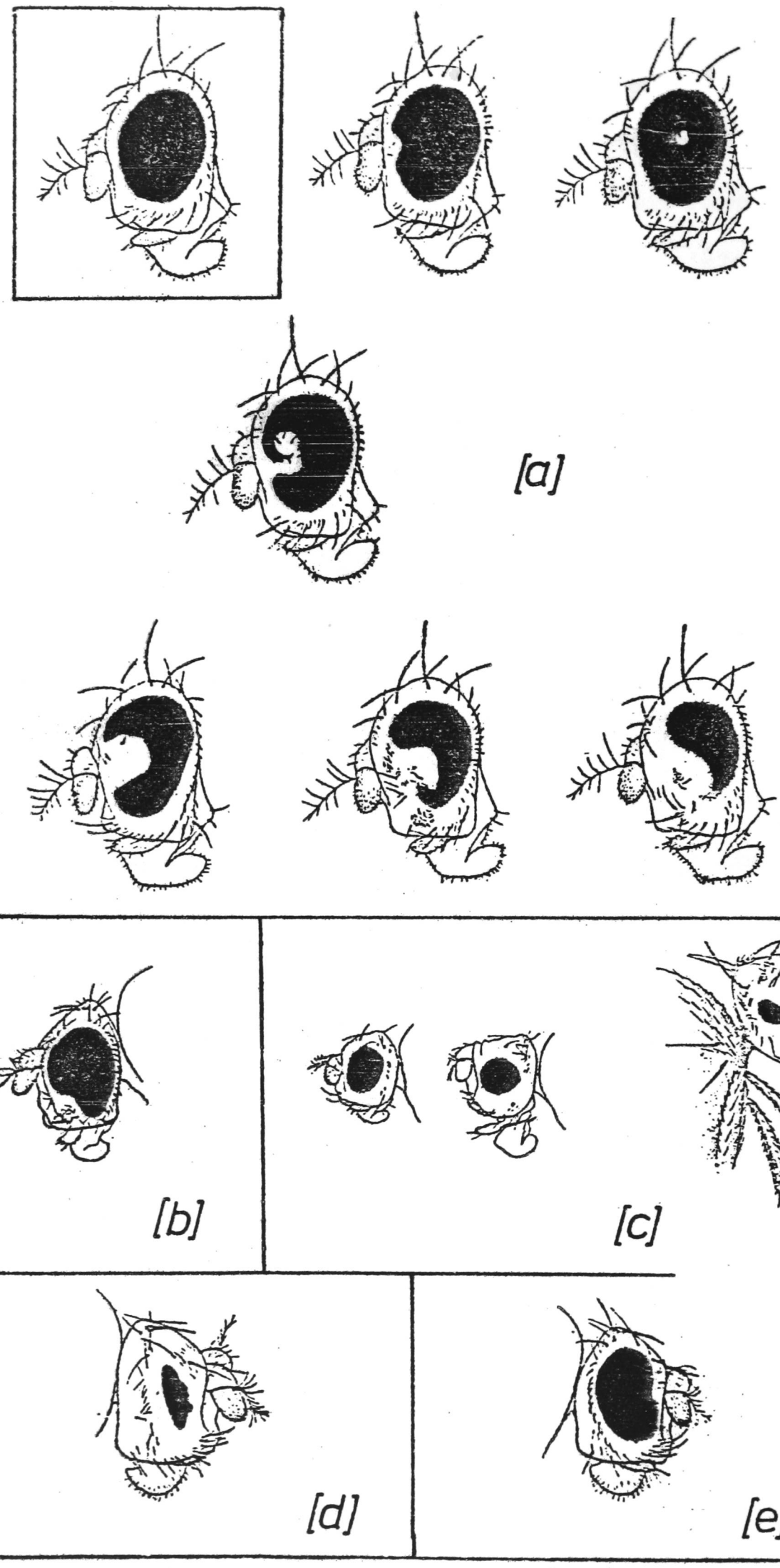


FIGURE 6.1 Different eye shape mutants of Drosophila. (a) (inset) wild type eye, (a) (other) examples of eye shapes obtained by Gottchewski and Ma (1937). (b) lobe, (c) three examples of eyeless, (d) bar homozygote, (e) bar heterozygote. All figures reproduced from Bridges and Brehme(1945) except (a) which are from Gottchewski and Ma (1937).

argues for autonomy of development of the bar cells.

Bar eyes also have a characteristic 'nick' in the centre of the anterior face of the ommatidial region. In the $B/+^{B}$ genotype this nick is manifested as the anterior infolding of a 'kidney' shaped eye. Gottchewski and Ma (1937) have shown an interesting mutant based on kidney with incomplete penetrance, which shows a variety of ommatidial perimeter shapes - these also show the anterior reduction, as do other mutants like lobe (see figure 6.1a).

Theories of the mode of action of the bar gene.

Theories of bar gene action can be divided into two groups. The first of these comprises theories invoking cell division inhibitions and rate of cell divisions. The simplest model is that 1 or more cell divisions are prevented at some time during embryonic or early larval development. This model would lead to an exponential dosage curve - reference to figure 6.2 shows that this is not the case: a plot of log ommatidial number against log dose also does not give the straight line that would be expected if this were true. A variety of workers have attempted to explain bar on these simple quantitative grounds, for example Hersch and deMarinis (1938) and Goldschmidt (1945). Goldschmidt attempted to show that such an argument held, and that "the relative quantity or potency of action of each mutant would be inversely proportional to the number of cell divisions". He calculated that the growth exponential $2^{9.6}$ held for the number of cell divisions to give about 775 'ommatidial precursors' starting from 2 initial cells in the wild type eye, and that the

doses of bar could be expressed by N/n, where N is a constant, and n is the exponent for giving the ommatidial number of the particular dose. Thus the wild type 'dosage' would be, if we arbitrarily set the constant to 10, 10/9.6 = 1.04. Because of dosage compensation Goldschmidt took each female bar dose to be contributing half of this figure, and the value for the 'dosage' in each female bar locus is, therefore, 0.52.

Goldschmidt used Sturtevant's (1927) bar data to calculate the expected n exponent figures from the relative ommatidial numbers, and compared them with his theoretically expected doses, based on the multiples of 0.52. The following table (6.1) shows his results, and my own compared figures using Goldschmidt's scheme with Andrews' (1970) data.

bar genotype	n obtained from Andrews' data	$10/_n$ (Andrews)	$10/_n$ (Goldschmidt)	calc. (dose x .52)
$+^B/Y$	9.5	1.05	-	1.04
B/Y	6.5	1.547	-	2.08
BB/Y	4.9	2.04	-	3.12
$+^B/+^B$	9.5	1.05	-	1.05
	-	-	1.05	
$B/+^B$	8.67	1.15	-	1.34
	-	-	1.18	
B/B	6.17	1.62	-	2.08
$BB/+^B$	5.48	1.83	-	2.16
		-	1.82	
BB/B	4.95	2.02	-	2.46
		-	1.92	
BB/BB	4.61	2.17	-	3.23
			2.18	

Goldschmidt calls the discrepancies between expected and actual ratios "small". Reference to the table shows that this is not so, especially if the male doses, which Goldschmidt does not seem to consider, are taken into account. In order to fit well to the exponential scheme, each male dose greater than 1 must also be ½ of the 'total' proportion. This is clearly wrong. If $+^{B}/Y$ has approximately the same number of ommatidia as $+^{B}/+^{B}$, then it is quite reasonable to suppose that one male dose of bar is equivalent to two female doses. However, by this reckoning, B/Y should have the equivalent of four female doses, and BB/Y should have the equivalent of six. If, this is so, expected and 'actual' values are quite different.

Rapoport (1940) has used a similar argument - that division of the bar eye cells is slowed by a 'substance' produced by the duplicated bar region on the X - chromosome. This theory is not compatible with Chevais' (1943) observation that the rate of growth of the bar cell complement is the same as for wild-type discs. There are other factors which do not agree with cell division inhibition or prevention mechanisms. The bar eye disc is, at maximum, about 25% less in size than the wild-type disc (Chevais, 1943). If this is the sum total of the 'cell division inhibition', then it fails to explain how the 90% lesser number of ommatidia appear in the adult bar eye. Clearly, something does affect the early cell division of the disc to give the small reduction in size seen in the bar disc, but the evidence suggests that this is only a small part of the total effect of the bar gene duplication on development of the eye.

Because of the lack of convincing evidence to explain the action of bar by a cell division mechanism, and because factors, like temperature shocks, and addition of a wide range of chemicals can give wild-type, or near wild-type eyes, we are faced with the possibility that some later point in development is crucial to the development of the Drosophila eye. We are brought to the 'differentiation wave' mentioned earlier in this section. Little work has been done on considering bar development as affecting differentiation itself. Andrews (1970) is the only source I have found.

Dosing data in bar and bar with eyeless-Russian.

For the models to be discussed below, I have used the data of Andrews (1970) and Rapoport (1941). This consisted of various data, from wild-type genotypes to octuple doses of bar. Andrews' data consisted of sets of dosing series on five different genetic backgrounds over the genotype range,

male

$+^B/Y$, B/Y, BB/Y.

female

$+^B/+^B$, $B/+^B$, B/B, BB/B, $BB/+^B$, BB/BB.

The genetic backgrounds were,

Eyeless Russian heterozygote background.

Gebarrus L.

Gebarrus 'control'.

Gebarrus H.

Eyeless Russian homozygote.

The Gebarrus strains used had been selected over a long

BAR GENOTYPE		STRAIN										Average props. of wild type.
		$+ey^R/-$		ey^R/ey^R		Gebarrus L		Gebarrus C		Gebarrus H		
		omm. nos.	prop. of WT.	omm. nos.	prop of WT.	omm nos.	prop. of WT.	omm. nos.	prop. of WT.	omm nos.	prop. of WT.	
♂	$+^B/Y$	718	1.0	485	1.0	688	1.0	744	1.0	782	1.0	1.0
	B/Y	88	0.123	33	0.068	86	0.125	92	0.124	96	0.123	0.1238
	BB/Y	30	0.042	10	0.021	29	0.042	33	0.044	35	0.045	0.0433
♀	$+^B/+^B$	742	1.0	501	1.0	745	1.0	793	1.0	847	1.0	1.0
	$B/+^B$	409	0.551	66	0.132	356	0.478	414	0.522	509	0.60	0.538
	B/B	72	0.097	25	0.050	70	0.094	78	0.098	90	0.106	0.099
	$BB/+^B$	45	0.06	12	0.024	41	0.056	46	0.058	49	0.058	0.058
	BB/B	31	0.042	9	0.018	29	0.039	33	0.042	36	0.0425	0.044
	BB/BB	24	0.033	8	0.016	25	0.034	27	0.034	29	0.034	0.0338
RAPOPORT'S DATA (1940)												
♀	$BBBB/+^B$	24	0.0307									0.0307
	$BBBB/BBBB$	8-12	0.012									0.012
	$BBBBBB/+^B$	17	0.0218									0.0218
	$BBBBBBBB/+^B$	4	0.005									0.005

TABLE 6.2 Actual ommatidial numbers and the proportions of the wild-type number that they represent. Data taken from Andrews (1970), and Rapoport (1940). 'Average' proportion column at right does not include the homozygous eyeless Russian data.

time for long thorax (Gebarrus H), short thorax (Gebarrus L) and also included a non-selected control strain. Andrews (1970) found that the ommatidial number in the adult was affected by the Gebarrus body size background, the following averages being for males carrying one dose of bar (i.e. $+^{B}/Y$).

Gebarrus L 688 ommatidia $\pm$ 0.70 s.e.

Gebarrus C 742 ommatidia $\pm$ 9.30 s.e.

Gebarrus H 781 ommatidia $\pm$ 6.50 s.e.

For comparison, the eyeless Russian heterozygote background had 718 ommatidia $\pm$ 1.15. The eyeless Russian homozygote was used by Andrews to look at the simultaneous influence of 2 mutants affecting eye size. The male $+^{B}/Y$ here had only 484.6 ommatidia $\pm$ 21.70 s.e.

Apart from this last set, all other dosing series give similar 'dose proportion' figures when converted to proportion of the wild-type dose. The table opposite (6.2) gives pooled results for the Gebarrus and eyeless Russian heterozygote data. The averaged figures from these tables may be plotted in the form of a graph of ommatidial proportion of wild-type versus dose. Several 'curves' can be drawn through the points. Andrews (1970) chose to plot two curves, one 'male' and one 'female'; this was done because of the difficulties of considering both sets of data together, due to dosage compensation.

Dosage compensation may be achieved by (i) a decrease of X-linked gene activity in the female; (ii) an increase of X-linked gene activity in the male; or, (iii) a combination of both mechanisms. Data on enzyme activities supports the first

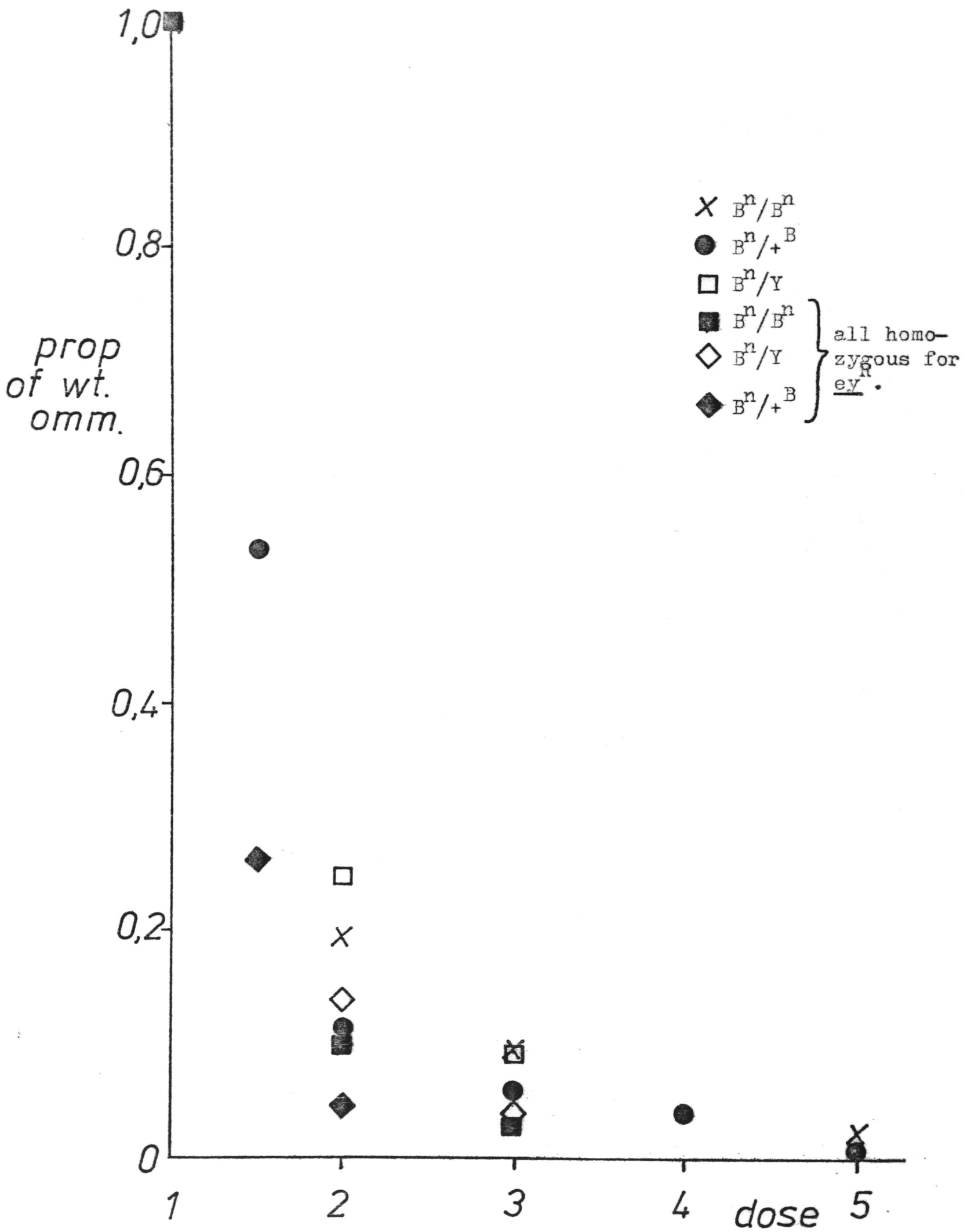


FIGURE 6.2 Graph of proportion of wild-type ommatidia per bar dose. Each female dose has been calculated as $\frac{1}{2}$ of the number of bar regions on the two X chromosomes, for example, B/B, with 4 actual regions, has the same dose ('2') as the male B/Y.

mechanism (e.g. Seecot, Kaplan and Futch, 1969), but cytological evidence suggests the second type of mechanism is in operation (Holmguist, 1972). Mukherjee, Lakhotia and Chatterjee (1968) have observed that the X chromosome in male larval salivary gland nuclei replicates faster than the paired X's in female nuclei or in the autosomes. There is, however, no definitive empirical basis for distinguishing between the alternatives.

In the graphs shown below, (figure 6.2) I have calculated each female dose as 0.5 of the male dose (= same as each male dose being 2 x female dose). In this way, two curves might be drawn. One would include all male hemizygotes and female homozygotes, and the other all female heterozygotes with one 'wild-type' chromatid. As the ommatidial proportions are so constant, even on different genetic backgrounds, we will consider only the $+ey^R/ey^R$ and ey^R/ey^R eyeless Russian/bar genes in the models to be discussed below.

MODEL SYSTEMS - BASIS FOR USE.

The pattern of cell division in the eye disc may be significant for the differentiation process. Both Becker (1956) in Drosophila, and White (1961) in Aedes have shown the presence of a posterior to anterior division wave. There is some evidence that in Aedes at least, there is some factor other than a simple cell division pattern travelling from posterior to anterior of the disc. A cell division ordering is only specified in time, and does not imply any interaction between the neighbouring cells. The experiments of White (1961), discussed earlier, with epidermal

implants suggest that some reaction between cells - either an active stimulus or local 'nudging' - may be necessary for cells to differentiate, and that the mere acquisition of competence to differentiate is not sufficient. If a constraining mechanism, like that described in chapter five is in operation in the eye disc, then this could possible lead to a differentiation switch. In such a case, the constraining surrounding parts of the larva may stop growing at the end of the 3rd larval instar, and thus produce a 'shock' to the growing disc cells, as they 'attempt' to divide inside the constricted region. If we look at the data so far set out it can be seen that it would be difficult to reconcile the regularity of the bar dosing series with the range of different constraints that would be necessary. The shape of the adult eyes of bar and the other eye shape mutants also seem difficult to explain by such a mechanism.

In Lawrence's Rhodnius cuticle experiments (1971), the state of each cell is hypothesised as being set by a diffusible morphogen. (Turing, 1952). It is possible that a similar mechanism triggers differentiation in the insect discs; the 'morphogen' would not have to be specific for any individual disc, and the influx of a high level of ecdysone during the 3rd larval instar could itself act as the stimulus. The source of this influx to the head disc could be along the optic nerve connection to the brain (the optic nerve enters the head disc at the posterior). The brain region is a source of hormone production, and the experiments of Gottchewski and Querner (1962) show that there is a flow of material from brain to head disc.

We will now discuss in detail two variations of a model explaining the differentiation 'switch' based on release of a morphogen. The first of these hypothesises varying levels of morphogen release, the level depending on the number of bar doses present, with cells having a preset threshold level below which they will not respond. The second variation suggests that bar affects an inbuilt threshold of response within each cell, and the externally set morphogen level is not affected by the bar gene duplication.

Questions can be posed:

(1) Can the shape and position of the bar eye ommatidial perimeter outlines be produced using the model?

(2) Can the numeric bar eye dosing series, in the form of ommatidial numbers, be produced by the model? Most important, does the model enable us to treat the bar data as a series with a linear effect per increase in dose? If not, can some other simple mechanism be hypothesised?

(3) Does the model also fit the eyeless Russian/bar data?

The Diffusion Model.

From microscopic observation, the approximate area of each head disc at 72 hours in the third larval instar is 0.24 mm width (dorso-ventral axis of adult eye) and 0.20 mm in length (anterio-posterior axis, not including antennal disc. These figures were obtained from measurements made on published micrographs (Rungger-Brandle and van Dijk, 1973). From Ouweneel's (1970) fate map, we could estimate the approximate size of the

determined eye field to be 0.15 → 0.20 width, and 0.1 → 0.15 mm length. The maximum length for an 'eye disc gradient' with posterior source would, therefore, be about 0.15 mm.

If the concentration of a morphogen is different at points along a one dimensional gradient, then we know that, given a constant 'source' and 'sink' the gradient will attain linearity after a certain time. Crick (1971) has expressed this statement in the following form. If the source holds the concentration there to C_o, and a sink is at point x = 1, and the concentration there is zero, if the diffusion constant is everywhere the same, then after infinite time, the concentration at any point will be,

$$C = C_0 \quad \frac{1 - x}{1} \qquad \ldots.(6.1)$$

where $0 \leqslant x \leqslant 1$

A determination of the time t required for this process may be made. The time cannot depend on the actual concentrations of the morphogen along the gradient because if all the concentrations are changed by a given factor, the diffusion flux which equalises the concentrations is changed by the same factor, and the equalization time, therefore, remains unchanged. The only physical quantities governing t are therefore D, the diffusion coefficient and L, the linear size of the region under consideration. The dimensions for these quantities are $[D] = cm^2/sec$, $[L] = cm$. The orders of magnitude of t are therefore given by

$$t \sim L^2/D \qquad \ldots.(6.2)$$

The time for equalization of concentrations in a space of size L is proportional to the square of L and is inversely proportional to the diffusion coefficient.

The formula can be re-arranged to give,

$$L \sim \sqrt{(Dt)} \qquad \ldots.(6.3)$$

which is equivalent to the "rough and ready rule" of Crick (1971), that L is less than or equal to $\sqrt{t}$. Using this formulation, and our calculations above, it would take about 0.4 hours or less for the gradient to be set up in the eye. Munro and Crick (1971) have shown that the time required to set up a gradient in a 3D sphere is approximately the same as the time required to set a gradient up in a 1D linear array. This suggests that we can use the approximation (6.2) in the case of the head disc which is more like the intermediate case of 2D diffusion from a point source into a plane sheet.

Munro and Crick (1971) have investigated the time a gradient would take to set up under a range of starting conditions. They found that if the morphogen level were everywhere zero, it would take 4 times as long to set up the gradient than if there was a starting concentration of half the maximum value. Munro and Crick also considered more complex models with varying flux, but the times required to set up such gradients were not greatly different than for the simple case.

Crick (1970) has argued that a reasonable diffusion coefficient for a morphogen with molecular weight about 500 (ATP has a molecular weight of 507, steriods are largely below 500: ecdysone has a molecular weight of 464) is about 0.3×10^{-6} cm/sec. This sort of value was found to fit for the hydra simulation discussed in chapter two, page 59 (Wolpert et al., 1972). Crick estimates that the gradient would be set up over 0.7 mm in 3 hours in this case. Our figure of 0.4 hours for the

smaller distance in the head disc seems, for this reason, that there are two types of 'morphogen' which could act on the head disc. The first would be a short lived pulse acting for a very limited time (less than 1 hour). The second would presumably be present for long enough to reach a steady state. As transplantation of the head disc into the abdomen of another larva can give 'normal' eye development (Bodenstein, 1939) it seems more likely that a more general, longer term stimulus is acting than a short pulse.

The distance of 0.15 mm appears rather small for a gradient in the sense of one imposing 'positional information' (most of the other examples quoted by, say, Wolpert, (1971) are systems with a heterogeneity of cell types in the 'field'). The axial length of Hydra is 2mm., the larval Rhodnius segment is 0.5 mm, and the chick limb bud at stage 24 is also 0.5 mm long. The width of the gradient in cell number is also low. If we take the disc to comprise of 700 - 1400 cells at the time of differentiation, this gives a figure of 15 - 20 cells, compared with 50 - 100 for the epidermal cells from front edge to back of the larval insect segment.

The first problem that arises in setting up a model of the diffusion of a morphogen in the head disc is one of area. Clearly, unlike the cases considered in Crick's (1971) simulations, diffusion would not be limited to one dimension, and the equations for such diffusion will not hold.

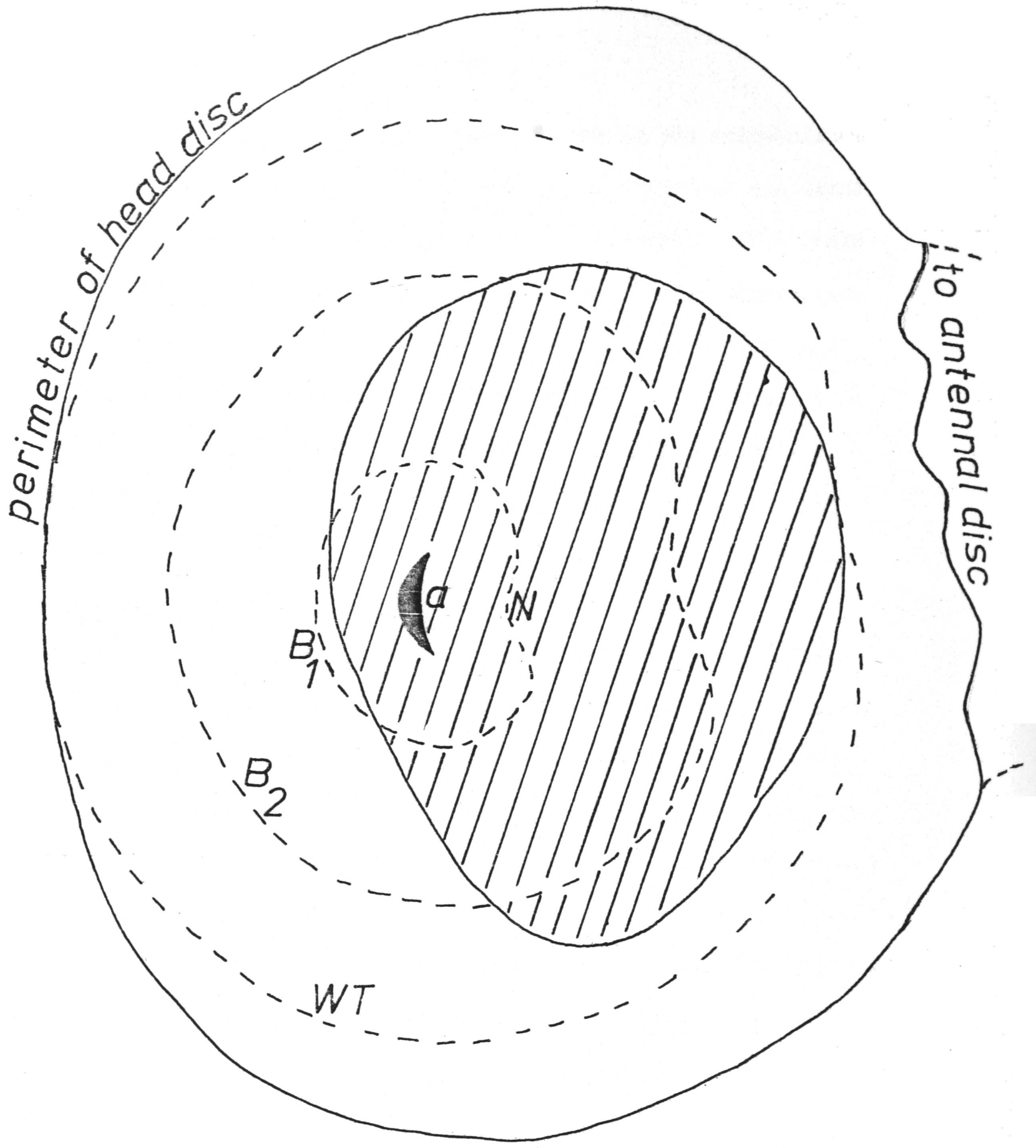


FIGURE 6.3 Simple morphogen threshold explanation of the bar dosing series. Crescent shaped area (a) produces or pumps morphogen. WT, B_2 and B_1 are the threshold radii of morphogen diffusion for wild-type, bar heterozygotes ($B/+^{B}$), and bar homozygotes (B/B) respectively. Hatched region is the extent of the ommatidial determined area.

Model (1). The morphogen level is changed by bar.

If we accept, as the basis of our model, that there is a source of morphogen in the head disc, and that some genetic variation in its production parameters affect the extent of ommatidial differentiation, either by affecting the rate, or flux of morphogen production, then we might set this 'source' as a region in the centre of the presumptive ommatidial region in the disc. We can estimate the location of the source by 'mapping' the adult eye, throughout the bar series, onto the head disc. If there is some kind of diffusion which activates differentiation from the source, and this diffusion is interrupted in some way by some bar gene property, then clearly, the bar eye has received a lesser morphogen flow. We can trace this through to multiple doses of bar with only 4 ommatidia (Rapoport, 1940). We will, therefore, take the source to be at the same relative position in the determined disc area as these low numbers of ommatidia appear in the adult eye.

Figure 6.3 shows how this scheme might be set out. The source, which may represent either the morphogen production site itself, or possibly the point of entry of morphogen into the eye region of the disc, must be curved as shown in order to give, by simple diffusion, the nicked (N) anterior of the bar ommatidial region seen in the adult fly.

We have already argued from Crick's calculations that, as the time for setting up a steady state in such a small area will be so short, the steady state case may be a realistic situation to study.

A two dimensional diffusion system of this sort, with a non-point source, cannot be easily handled in terms of diffusion equations. A far simpler case, which we will consider here, would be diffusion across a hollow cylinder, whose outer and inner radii are a and b respectively (this case has been worked out by Crank, 1956). As our head disc morphogen diffusion takes place from a set source into a two dimensional area, we can equate the theoretical case of the cross section of the cylinder with the morphogen diffusion. In the cylinder, the <u>steady state</u> condition is,

$$\frac{d}{dr}\left(r \cdot \frac{dC}{dr}\right) = 0 \quad \text{where} \quad a < r < b \qquad \ldots.(6.4)$$

and the general solution is,

$$C = A + B + \log r \qquad \ldots.(6.5)$$

where A and B are constants.

Details of the model.

We define a two dimensional circle of radius R, which represents the 'maximum limit of morphogen diffusion'. In the centre of this circle is another circle of radius a which represents the extent of the source. The concentration of morphogen at the source is set to a level c = C, and the concentration at radius R is held at c = 0.

If D = diffusion coefficient of the morphogen, the flux throughout the system will be constant at,

$$F = -2\pi r D \frac{\delta c}{\delta r} \qquad \ldots.(6.6)$$

therefore,

$$F = -2\pi r D . B \frac{1}{r} \qquad \ldots.(6.7)$$

$$= -2\pi D.B \qquad \ldots.(6.8)$$

$$\therefore \quad B = -F/2\pi D \qquad \ldots.(6.9)$$

If $c = 0$ at $r = R$

$$0 = A - {}^{F}/_{2\pi D} \cdot \log R \qquad \ldots(6.10)$$

$$A = {}^{F}/_{2\pi D} \cdot \log R \qquad \ldots(6.11)$$

Now,

$$c = {}^{F}/_{2\pi D} \cdot \log \left({}^{R}/_{r}\right) \qquad \ldots(6.12)$$

and, hence

$$e^{\,{}^{c}/_{(F/2\pi D)}} = {}^{R}/_{r} \qquad \ldots(6.13)$$

or,

$${}^{r}/_{R} = e^{\,{}^{-c}/_{(F/2\pi D)}} \qquad \ldots(6.14)$$

We can now define distance r_{τ} to any threshold concentration (as shown in figure 6.3) $c = \tau$ as,

$${}^{r_{\tau}}/_{R} = e^{\,-{}^{\tau}/_{(F/2\pi D)}} \qquad \ldots(6.15)$$

In order to test the model we must fit it to the bar series data and see if a feasible kind of reduction in flux at the source can give predictions fitting with these data. The ommatidial counts obtained from the bar series must be transformed into equivalent areas (in the disc) so that these numbers can be related to our diffusion radii. A simple procedure was devised by which the two could be approximately equated.

A two dimensional lattice, with the points arranged in a hexagonal format was used. A 'determined area perimeter' was defined on this space so that it represented the approximate outline of the third instar determined ommatidial area. The size of the space was chosen to include inside it the number of lattice points equivalent to the total ommatidial number in

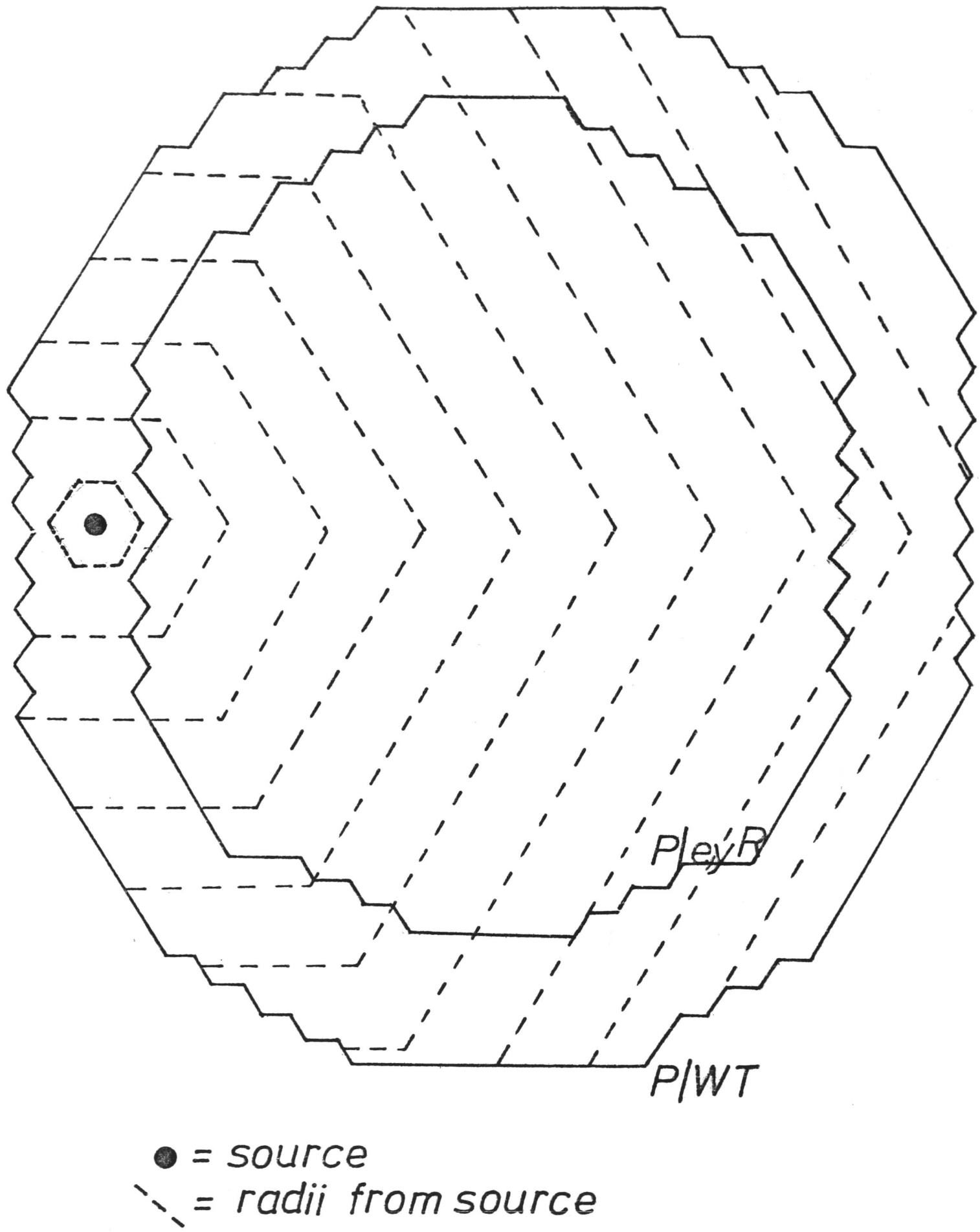
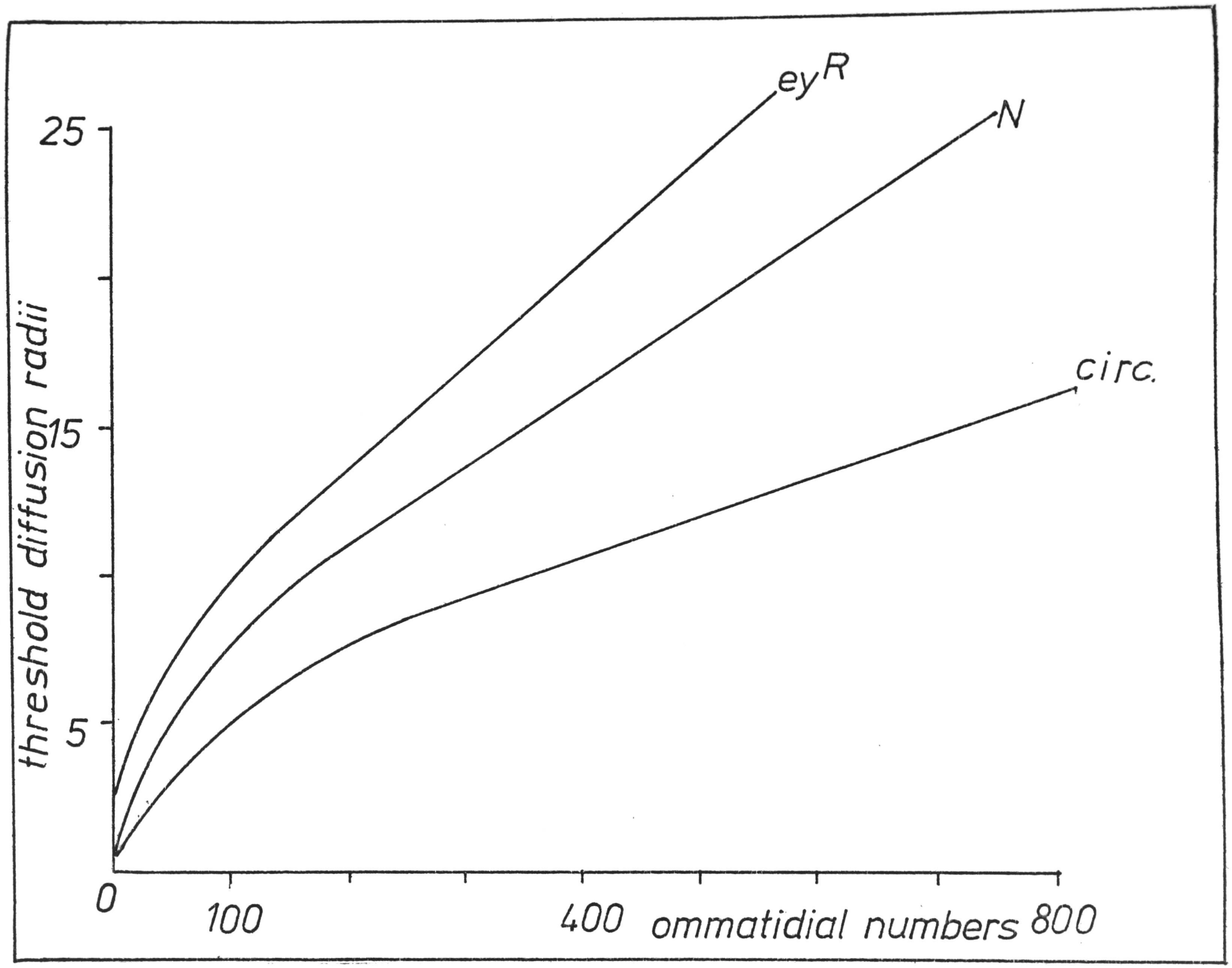


FIGURE 6.4 Transformation of ommatidial areas into distances to morphogen thresholds. P/WT = perimeter of wild type or bar determined area. P/ey^{R} = equivalent perimeter of eyeless Russian determined area. Distances from the source were measured, and number of 'ommatidia' within each radius from the source were calculated.

FIGURE 6.5 Transformed data of previous figure. Graph of threshold diffusion radii, measured in 'ommatidial diameters', against ommatidial number. N=curve for wild-type determined area. ey^R = curve for reduced eyeless Russian determined area. circ.= curve drawn for comparison for areas and radii calculated from a point source in the centre of the determined area P/WT of the last figure.



GENOTYPE		BAR DOSE (each ♀ dose = ½ ♂ dose)	Omm. Nos.	Omm. prop. of WT.	'Transformed' r_T values
bar					
♂	$+^B/Y$	1	718	1·0	25·0
	B/Y	2	88	0·123	7·2
	BB/Y	3	30	0·042	3·6
♀	$+^B/+^B$	1	742	1·0	25·6
	$B/+^B$	1·5	409	0·551	16·7
	B/B	2	72	0·097	6·4
	$BB/+^B$	2	45	0·06	4·8
	BB/B	2·5	31	0·042	3·6
	BB/BB	3	24	0·033	3·1
	$BBB/+^B$	3	24	0·0307	3·1
	BBB/BBB	4	8-12	0·012	1·8
	$BBBB/+^B$	5	17	0·0218	2·2
	$BBBBB/+^B$	5	4	0·005	1·0
bar/eyless Russian					
♂	$+^B/Y$	1	485	1·0	23·8
	B/Y	2	33	0·068	5·6
	BB/Y	3	10	0·021	3·6
♀	$+^B/+^B$	1	501	1·0	24·3
	$B/+^B$	1·5	66	0·132	7·5
	B/B	2	25	0·0499	5·1
	$BB/+^B$	2	12	0·024	3·6
	BB/B	2·5	9	0·018	3·5
	BB/BB	3	8	0·016	3·4

TABLE 6.3 Shows r_T, distance to supposed threshold concentration of morphogen, for the $+ey^R/-$ and ey^R/ey^R background bar dosing series. r_T values have been read off figure 6.5.

the wild-type adult eye - between 700 - 850 points.

One of these points was chosen as the 'source' position, equivalent to the source in figure 6.3. The simplified 'point' source was chosen because it was closer to the theoretical diffusion situation considered above; curved sources were also considered, and gave roughly the same quantitative results. The number of 'activated' ommatidia were then found for each successive 'cell radius' out from the source point (figure 6.4). A graph of number of radii against activated ommatidia could then be drawn (figure 6.5). This graph shows three curves: N is the point source we have considered, circ is the curve produced if the point is initially situated in the centre of the determined space and eyR is the curve for the limited determined area of the 'eyeless Russian eye' (see reduced perimeter in figure 6.4).

We can use the N curve to read off the maximum effective diffusion radius for each dose of bar. For example, 700 ommatidia is equivalent to a radius of about 24. In this way, we can obtain our experimental equivalent figures to the r variable in equation (6.15). The values obtained are shown in table 6.3.

Now, if (6.15) is true, then, by taking logs.,

$$\text{Log } (r_\tau) = \log (R) - 2\pi D\tau^{1}/F \qquad \ldots(6.16)$$

If F, the flux, is inhibited by the bar gene product, assumed to be proportional to dose, we may enquire whether some simple 'inhibition function' would satisfy the observed data. Two such functions are the familiar 'competitive' and 'non-competitive' inhibitions. Both can be represented by an expression

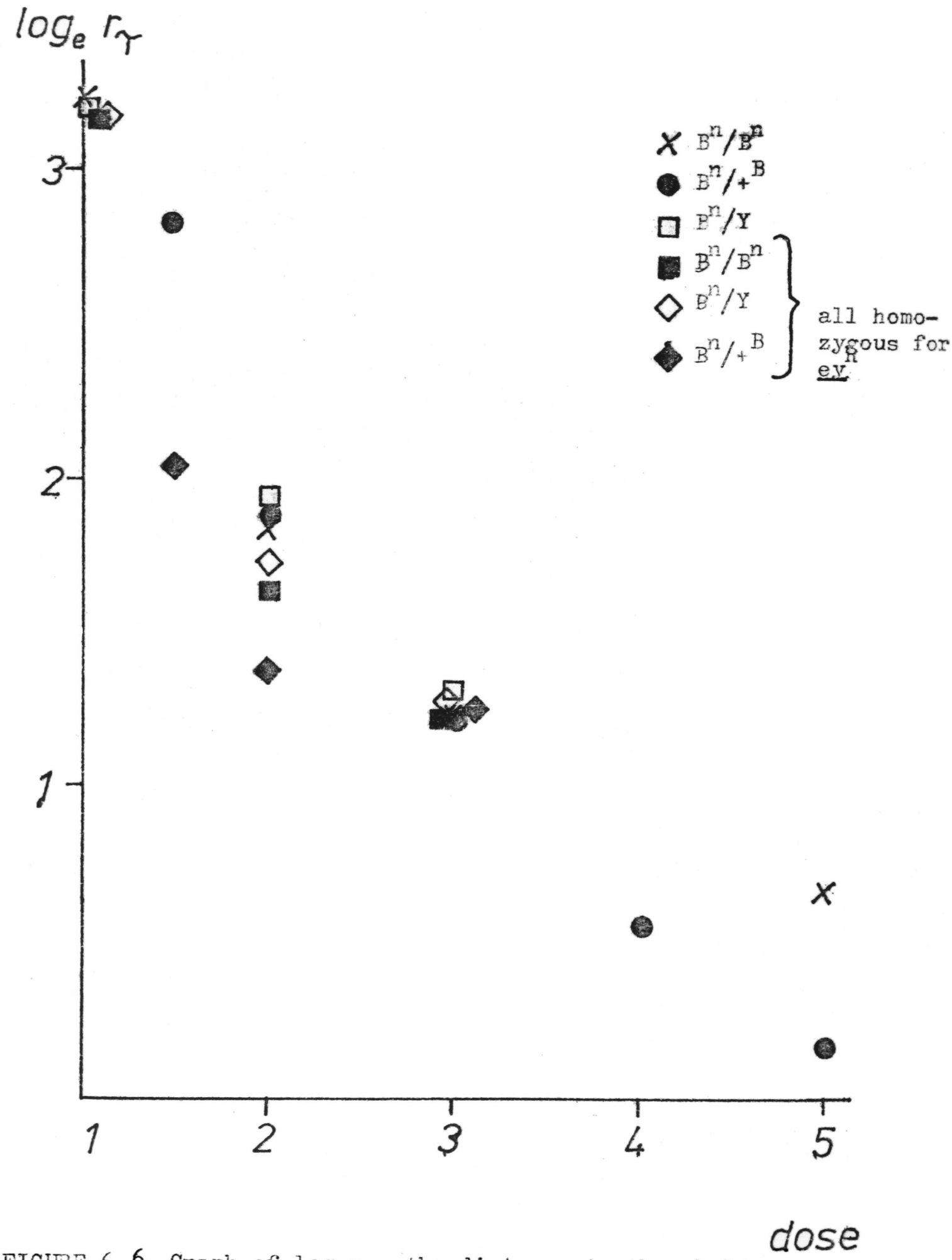


FIGURE 6.6 Graph of $\log_e r_\tau$, the distance to threshold concentration of morphogen, against dose. Dose is calculated in the same manner as in Figure 6.2, with each 2 female bar regions counting as the equivalent of one male bar region.

$$1/F = A + Dd \qquad \ldots(6.17)$$

where d = dose of inhibition

this gives,

$$\log r_T = A' + B'd \qquad \ldots(6.18)$$

and means that a plot of log r_T against bar dose, d should give a straight line. Such a plot is given in figure 6.6. It shows that the simple inhibition hypothesis does not hold, as the data cannot be easily fitted to a straight line.

It is, of course, possible that some more complex inhibition behaviour operates but in the absence of any information on this we must consider the proposed mechanism as unlikely.

Model (2). The individual cell morphogen threshold is changed by bar.

There are two arguments against the validity of the morphogen inhibition model. The first is that the 'inhibition function' is not one of the well known, 'Michaelis' or 'non-competitive' inhibition types (nor would if easily fit an allosteric function). The second is the evidence, discussed earlier, that bar cells show autonomy during development (deMarinis, 1952; Steinberg, 1941; Sturtevant, 1927), i.e. that the 'bar' property is inherent in the cells of the disc and not in some 'signal' generated from elsewhere. Our morphogen scheme must be ammended to fit with their criticisms, and this amendment will be considered here.

Retaining the "morphogen/threshold" relationship as the basis of our model, we now investigate the properties of a model

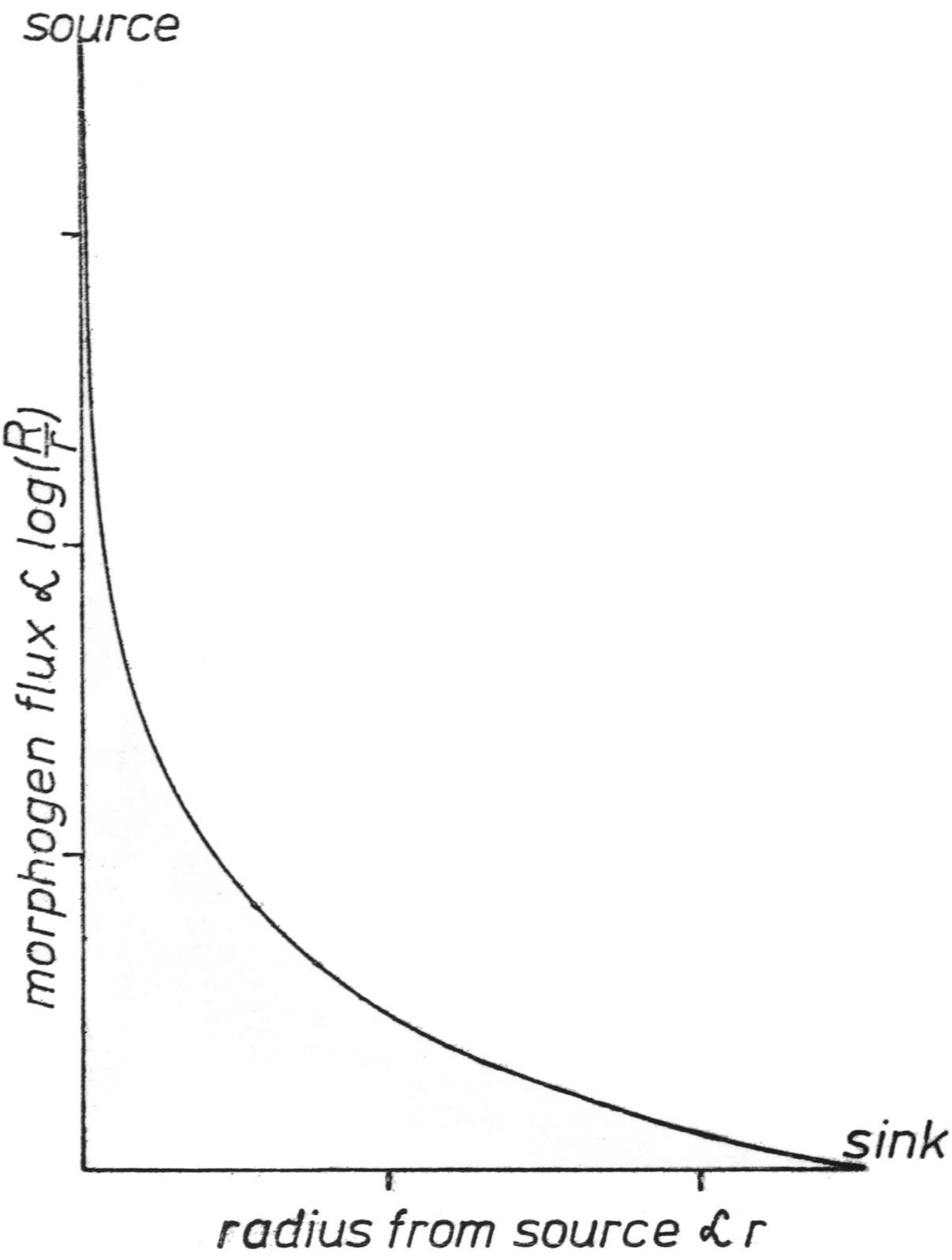


FIGURE 6.7 Graph of log (R/r) v. proportional distance from source to sink. Shows shape of concentration distance produced using equation 6.12.

that has a constant morphogen supply but whose cells respond to the level of morphogen according to their bar genotype. This means that the threshold concentrations are genetically determined and are dose dependent. It can be seen from equation (6.12) that the concentration at any point r along the gradient will be proportional to log $^{R}/_{r}$, where R is the distance from source to sink. We can therefore plot a graph (figure 6.7) which shows how, using the model, the concentration will fall as we travel away from the source. It can be seen that cells situated along this radius will be exposed to different concentrations of the morphogen and therefore, should a threshold value for differentiation exist, a limit to the cells transformed will be manifested. Different cell types, having inherently different threshold responses will, therefore, generate different areas of ommatidial differentiation. In figure 6.5 we have estimated the 'radius' of the action of an assumed morphogen giving the ommatidial areas produced starting from a peripheral source (figure 6.4). These radii are in arbitrary units ('ommatidial diameters'). The scale of the radius in figure 6.7 is also arbitrary, being given in terms of fractions of $^{R}/_{r}$. It is, therefore, possible to map the number of ommatidia in the series onto the threshold values of the morphogen flux. Since the ommatidial numbers are generated by a regular series of increasing doses of the bar region, we end up with a plot of dose against threshold concentration of morphogen (table 6.4 and figure 6.8). It can be seen that this transformation gives a reasonable straight line for both the normal and the eyeless-

BAR DOSE (each ♀ dose = ½ ♂ dose)		threshold concs. for set r_{WT}	'Transformed' r_T values
bar			
♂	1	1·39	25·0
	2	2·60	7·2
	3	3·28	3·6
♀	1	1·36	25·6
	1·5	1·80	16·7
	2	2·74	6·4
	2·5	3·28	3·6
	3	3·45	3·1
	3	3·45	3·1
	4	4·06	1·8
	5	3·86	2·2
	5	4·64	1·0
bar/eyeless Russian			
♂	1	1·43	23·8
	2	2·85	5·6
	3	3·28	3·6
♀	1	1·41	24·3
	1·5	2·60	7·5
	2	2·88	5·1
	2	3·28	3·6
	2·5	3·32	3·5
	3	3·36	3·4

TABLE 6·4 'threshold concentrations' for r series along the X axis in figure 6.7, taking r_{WT} as the r_T of the wild type.

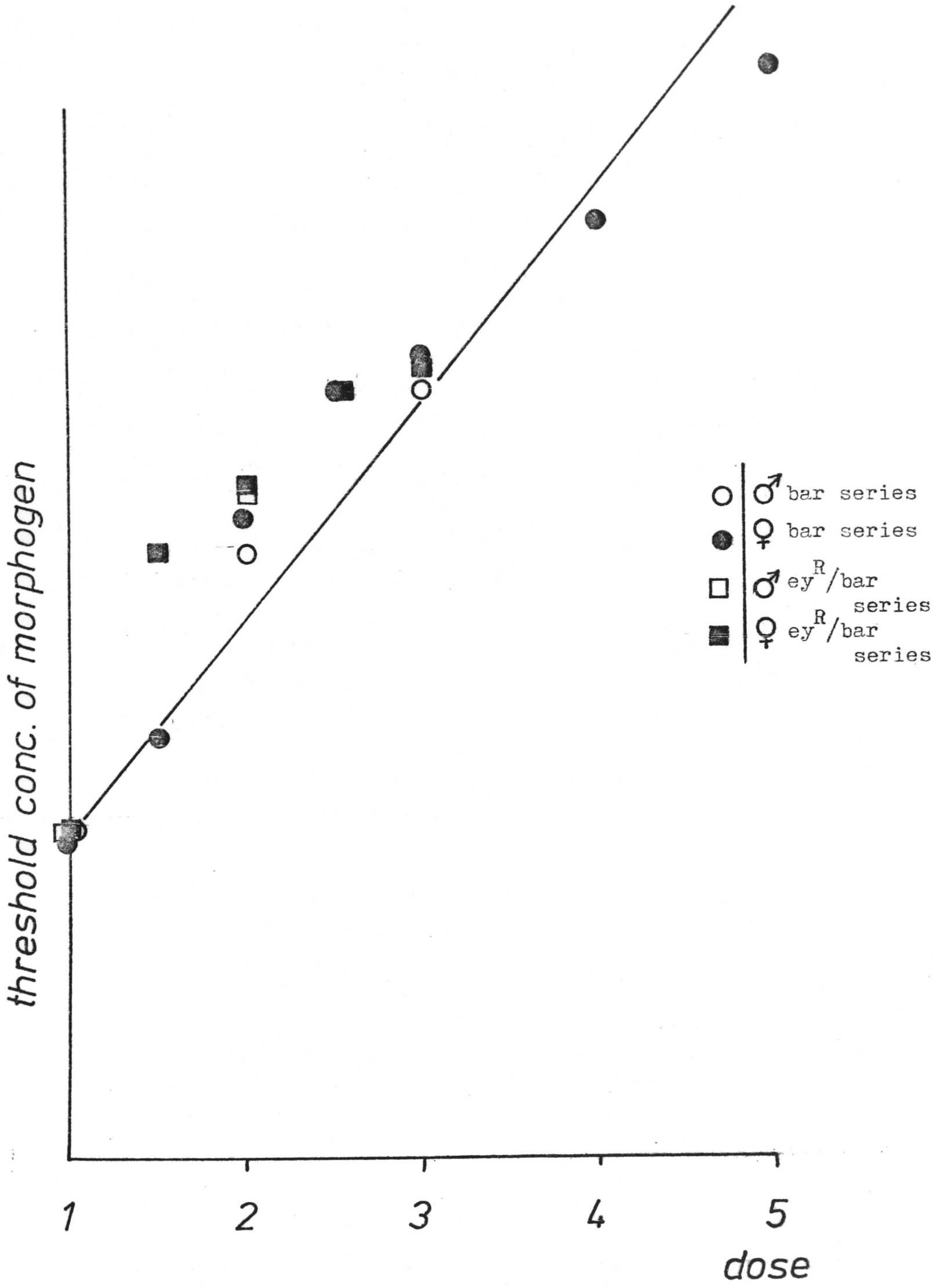


FIGURE 6.8 Graph of dose v. threshold concentration of morphogen. Threshold values are on an arbitrary scaling as on the previous figure, and were obtained by finding the 'concentrations' for the various r_γ points in table 6.4.

Russian data including fairly large doses. It therefore appears that the assumption of bar affecting the threshold towards which the cells react is a reasonably satisfactory prediction for the number of ommatidia finally generated. The non-linear relationship between dose and final ommatidial number is therefore seen to be due to two factors. (1) the assymetry of the source of morphogen in the cell mass, and (2) the non linear diffusion gradient set up from this source. The bar 'gene product', on this model, is therefore seen to affect in a linear manner the response of the cells to the differentiation signal which floods through the tissue. What the mechanism of this change of response is can, of course, not be specified from this analysis. The mechanism is of an antagonistic kind, but since little is known of the chemistry of differentiation in response to external signals, it would be futile to speculate any further.

A HYPOTHETICAL GENERAL DIFFUSION MODEL OF EYE DISC DIFFERENTIATION IN BAR AND THE NORMAL EYE.

1. Early in development, a certain number of cells are determined to form the two head discs. As time progresses, each disc becomes determined into the various individual regions, and forms the 'mature disc' with its full complement of cells. At this time (third larval instar) it is ready to differentiate.

2. When the larval disc is mature, a 'morphogen' is released from either the brain or optic glomeruli.

This morphogen may be ecdysone; it diffuses across the eye disc and 'switches on' the presumptive ommatidial cell differentiation.

3a. In the bar series the source of the morphogen is diminished, and fewer cells are therefore switched on. The relative distances diffused by the morphogen by each dose of bar are related in a complex inhibitory manner.

3b. Alternatively, each cell in the disc has its differentiation threshold increased with each increase in 'bar' dose in a linear manner.

4. The point of entry of morphogen into the eye disc is crescent shaped. This does not affect the shape of the full eye, as all determined cells are successfully switched on. (See figure 6.3 patterns).

5. If all cells are not switched on, the crescent shaped initiation centre leads to an ommatidial perimeter with a 'nick' in the front edge.

CHAPTER SEVEN

GENERAL CONCLUSIONS

We must now look, in general terms, at the success of the approaches set out in the preceding chapters. There are several important points to be made. Firstly, the various techniques and methodologies of use in studying developmental models are diverse, and no attempt has yet been made to bring them together into an overall scheme. The few existing studies of such models have been under such essentially restrictive headings as 'morphogenesis' or 'cellular automata', and have dealt either with the author's own model system (e.g. Arbib's book 'Theories of Abstract Automata' (1969), or with one particular aspect of development (Rosen's review of theoretical aspects of the 'self assembly' side of morphogenesis (1972). It is hoped that chapters 1, 2 and 3 of this thesis provide something of an introduction to the field of computer models in developmental biology, and so partly rectify the gap between 'computer manual' and 'biological model' that has existed in the past.

It is necessary, when dealing with a basically theoretical topic, to show that the methods studied can be applied to give useful advances in knowledge, and are not just 'models for the sake of models'. In the first chapter it was stated that the models most amenable to computer study are those in the heuristic (Kacser, 1960) category. The application of computers, within the confines of this thesis, have also been further limited to those heuristic models involving the interaction of large numbers of 'simple units' (cells). The second part of the thesis, has been

largely concerned with describing the use of various heuristic models using computers.

The first model described the analysis of patches and clones in chimaeras and X-inactivation mosaics. A basic question asked was - Can we, given a final adult chimaeric tissue, predict data about an earlier time in the tissues' development? A central problem to which such an approach is geared is, of course, that of trying to discover when X-inactivation takes place in mammals. A model was designed which, it is hoped, can help to answer such questions.

The other work included in this thesis was based on studies of the development of the Drosophila imaginal head disc. In the first imaginal disc model, various parameters important in the formation of clones produced by somatic crossing over or position effect variegation in the eye were considered. It was shown that previous observations about the shapes of these clones can be explained with the aid of a computer model. The 'rules' for growth in the model were analysed, and used to suggest what forces are acting in the living organism to produce the modelled phenomena. The results indicated that boundary forces acting on the disc could produce the oriented clones seen in the practical work of Becker (1957). The physical nature of these forces was discussed: it was also suggested that the change in division orientations hypothesised by Becker to occur at the end of the first larval instar is not a change as such, but the channeling of direction from random to constrained division. This model may be of importance in suggesting how shapes can be generated

during development by the morphogenesis of masses of cells without mediation of active gradients of the type discussed in chapter one.

A rather different situation is provided in the later development of the head disc. Here, there seems to be a defined 'switch' at the onset of the third larval instar which controls the capacity of the determined disc cells to differentiate. Models were described which suggest how the quantitative reduction in ommatidial number produced by the bar duplication series may be explained. As it is suggested that these models affect the differentiation process, some clues as to the mode of action of the 'differentiation switch' was provided.

The interesting, although tentative, conclusion of this work is that there is some active chemical switch which provides the stimulus for differentiation to start. Although I have argued earlier in this thesis that the importance of chemical gradients has been overstressed in the last few years, partly as compensation for the point of view put recently by Crick (1970) ("An outsider to embryology has the impression that in recent years, gradients have become a dirty word"), this apparent reversal of point of view is only partial. There are two reasons for this. Firstly, the 'morphogen' would only have to exist in the form of a 'greater than threshold level'; no vectorial position recognition is needed; there is also the precedent that known chemical substances, especially ecdysone, have been shown to be vital for differentiation.

Finally, the conclusions of the first chapter might again be stressed. Talking about the responses of cells to their environments in the general terms of 'gradientology' is to become

confused about the cause and effect of embryological events. We have shown how, for example, a 'gradient' in cell division direction clearly exists across the model of the developing eye disc, but this vector is imposed by both intrinsic and extrinsic mechanisms, and may not be best considered in gradient phraseology.

I have tried to show how 'cellular' systems may be modelled, and how the observance of the properties of the models may allow us to assess the assumptions made in setting up such models. In this way we can check back to the living system and produce, in the words of Braverman and Schrandt (1967) "a continuous dialogue between question and suggestion".

APPENDIX

VIDEO TECHNIQUES

DISPLAY OF OUTPUT

The reader will probably be familiar with the variety of output devices that can be coupled up to a computer. These consist, for the main part, of line printer, teletype, graph plotter and video displays. Unfortunately, it may not be easy, especially with a new program, to work out the usefulness, and time and cost-effectiveness of particular devices for particular simulations. The following scheme shows an ordered schedule actually used in the running of a simulation program. It may help to illustrate how the various output devices may be most profitably used in developing a model of this sort.

1. Model worked out and described (pencil and paper).
2. Program written, and preliminary form punched on paper tape.
3. Program fed into multi-access system, and debugged using teletype, with teletype output.
4. Teletype operation for program development, line printer output.
5. Program amended and transferred to video system, using small dedicated computer. 'Behaviour' of program observed.
6. Program run using batch process on large computer for large numbers of results.

Video has distinct advantages over hard-copy devices like line printer, graph plotter, or teletype. These latter can only print 'time slice' samples of the progress of a simulation. The

video screen, on the other hand, allows us to watch everything that is happening, as any changes in array element values can be simultaneously updated on the screen. It is even possible to make cine films of simulated morphogenetic movements (Ransom and Lucey, 1972).

ADVANTAGES OF VIDEO TECHNIQUES: A NOTE ON INTERACTION

Besides giving greater accessibility of program to programmer, video also allows the use of interactive techniques. Using a computer interactively implies that man and machine are in some sort of feedback loop - what the machine produces influences the next set of data fed in by the user. In the most literal sense, almost every use of the computer could be considered interactive, even the act of compiling an incorrect program, which will require re-submission after the user has examined the diagnostic information produced by the compiler. But, to refine the definition further, we can distinguish the interactive program by its requirement for user intervention during the course of execution, in response to the information it has produced.

It is possible to halt the video program at any point during its running and make changes in the programs execution - for example, cells might be 'grafted' or 'removed' from a growing cell population, this will be discussed in more detail below.

There are a large number of video screens on the market, falling into two main categories - cathode ray tube (CRT) systems (refresh displays) working on the same principle as the TV screen,

and storage tubes. The storage tube has a 'gun' which emits a net positive charge onto the regions written on the screen (made of phosphor) - this charge is maintained and seen by the action of a 'flood gun', flooding the screen with a uniform pattern of electrons. In this case individual points on the screen cannot be changed or overwritten, and the screen has to be wiped afresh before new information can be introduced.

Input to video devices.

Both light pen (refresh display) and joystick (the equivalent mechanism on the storage tube) can be used, together with standard input facilities.

USING A REFRESH DISPLAY WITH A CELL POPULATION GROWTH PROGRAM

This is quite straightforward, although dependent on the state of the software facilities of the particular computer coupled to the CRT. The main difference between a program used with conventional output, and the program devised for video is the presence in the video program of a 'display file'. This consists of a modified array, basically similar to the main array of the program, used to mark the positions of the 'cells'.

The array is enlarged and modified in order to contain not only cell positions, but also control instructions for driving the video. These instructions have to be inserted into the correct places in the array, and so they make it an asymmetrical structure.

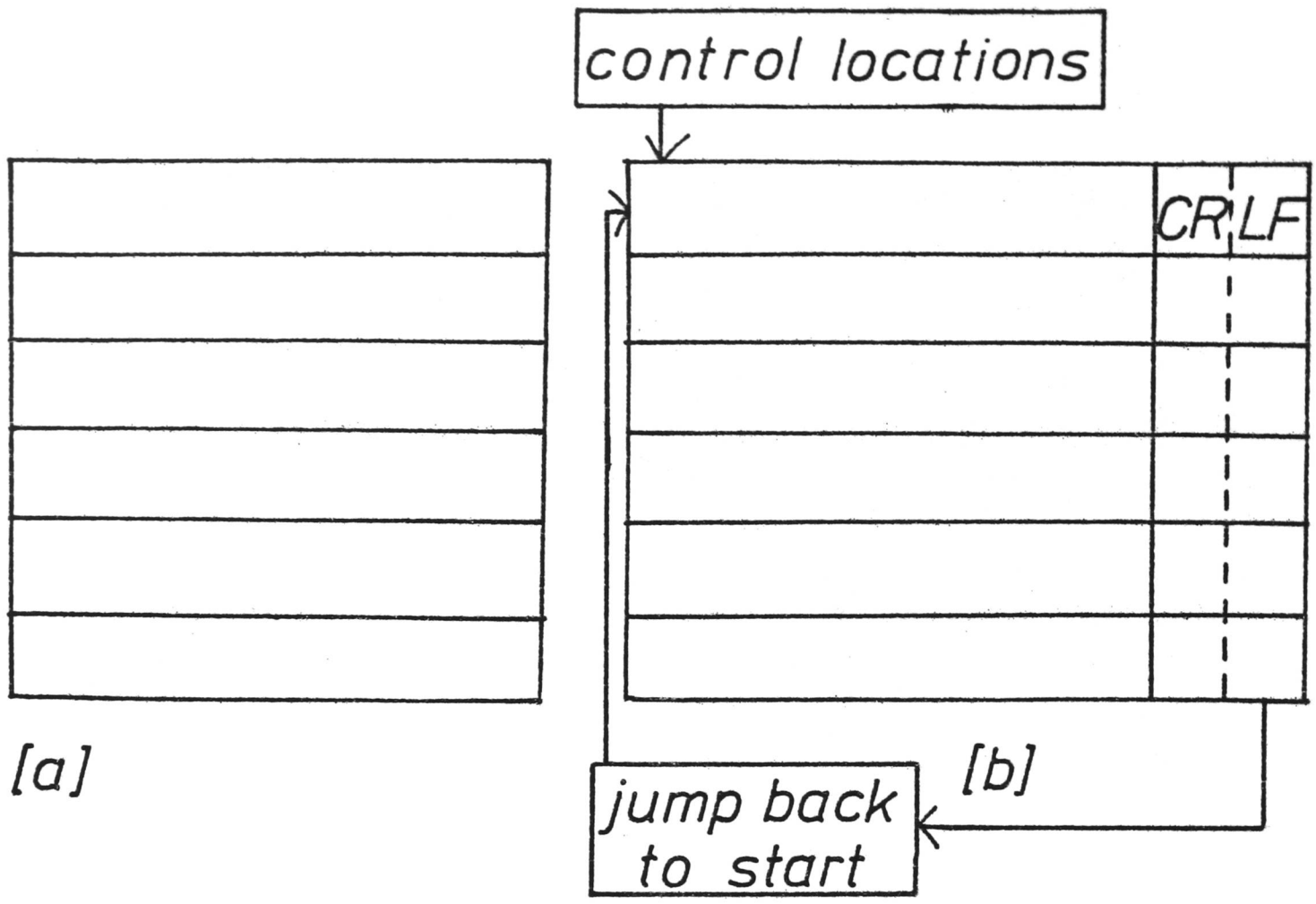


FIGURE A.1 Construction of video system. (a) Main, and (b) modified video arrays. CR = carriage return, LF = line feed. (c) Computer configuration used for video. The display file picks up information from the modified video array in the program: the display logic transforms this information into the electronic impulses required to drive the video. PC = program counter, DAC = display address counter.

Array positions 1-5 are used as control words to keep the refresh display cycling. At the end of each block of elements constituting a 'row' of normal array positions (c.f. figure 3.5) 2 extra array positions specify 'carriage return, line feed' for the raster. A final array position at the end of the program makes the display processor (the computer hardware linking display file with screen) loop back to the beginning of the file and repeat the process over again. Meanwhile, array elements in the main array may have changed value, and the changes are immediately transmitted into the modified array and hence to the screen. Figure A.1 shows a comparison between main (a) and modified arrays (b), and figure A.1C is a sketch of the computer configuration used.

The computer video processor, therefore, constantly samples from the display file, and updates the information on the screen. It is possible to use either characters from the standard computer character set, or else write special subroutines to generate shapes drawn by vectors. Details of the latter are probably best gleaned from the appropriate computer manual, figure A.2 shows a step in a video representation of a cell clone growth simulation using two special shapes to indicate two types of cells.

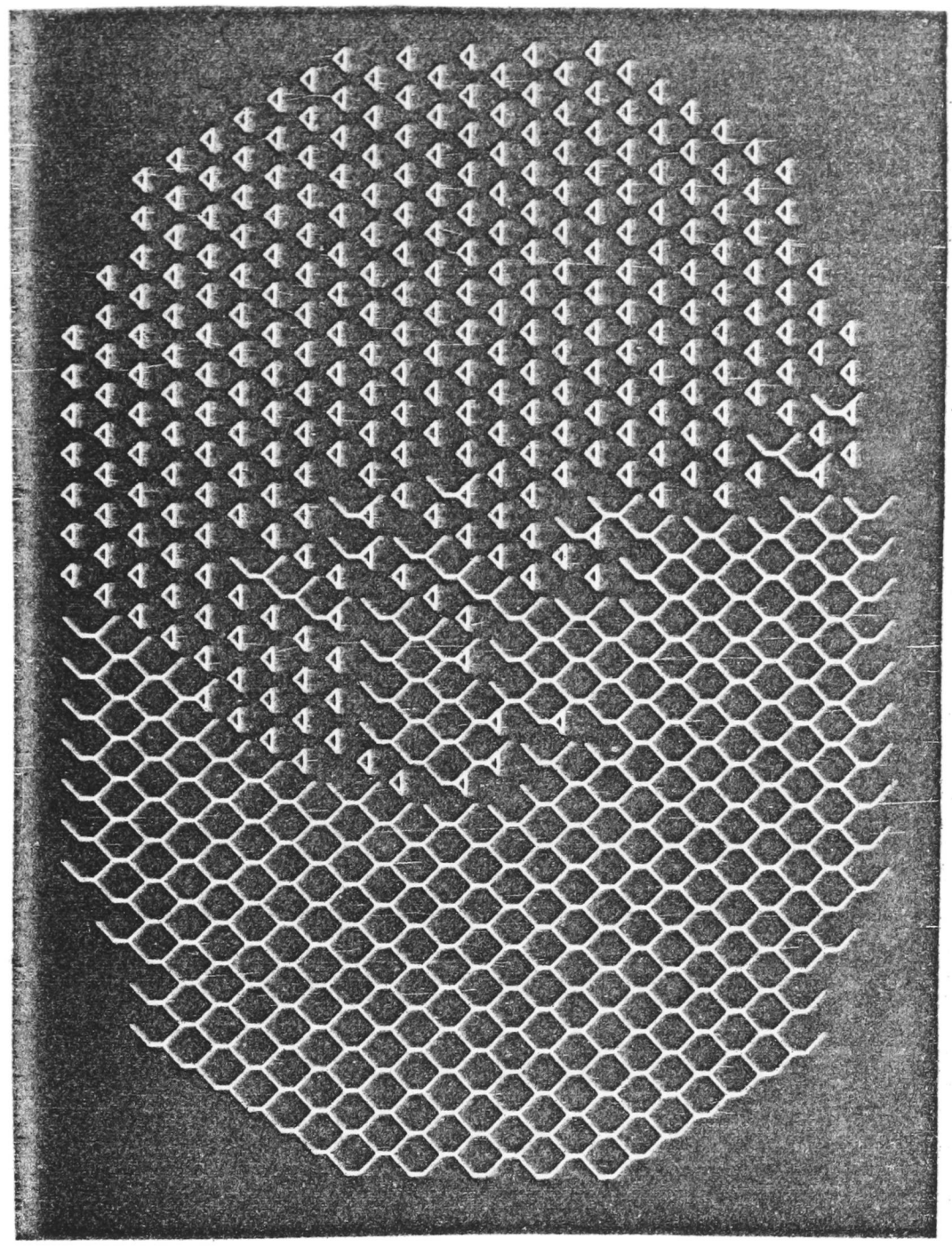


FIGURE A.2

Use of the light pen as an input device has already been discussed. It is also possible to spatially communicate with the program by means of the light pen. We have seen in chapter 3 that the use of arrays gives us an easy method of keeping track of the co-ordinates of simulated cells: by pointing at particular array positions displayed on the screen with the light pen, the computer can recognise the light pen's position - groups of cells or individual cells can thus be removed, put onto the screen or moved in position.

The video console is usually supplemented by a row of push buttons, and these can be implemented at any point during the running of the program, their effect being to activate a flag in the display processor. This flag is tested for during the running of the program by using a conditional instruction of the sort 'if PB 3 (push button) is pushed go to n', where n is a label. Push buttons are used in conjunction with the light pen - they can be employed to 'freeze' the video ready for the light pen to be used - or to directly interact with the program in some other way. A typical interaction step might occur as follows. The operator decided to remove a group of cells from a growing mass. The picture is 'frozen' by pushing a chosen push button. All 'cells' to be removed are pointed at with the light pen, and then these 'tagged' cells are removed by pushing a second push button. A third push button then restarts the growth process.

PROGRAMMING THE LIGHT PEN

The X and Y co-ordinates of the characters representing 'cells' on the screen are not important to the programmer when he is using the computers' standard character set. Each character will be drawn with the beam ending up in the correct position to draw the next character; it is only necessary to get to the beginning of each new line at the correct point. However, when the light pen is in operation, these co-ordinates become very important. A picture is generated on the screen by the passage of a stream of electrons onto the phosphorescent surface of the screen, and the beam traverses each row from left to right starting

at the top left hand corner of the screen, completing one picture in about 1/50th of a second. At any instant in time the beam will be at a particular set of X,Y co-ordinates on the screen. These co-ordinates are also 'known' to the computer, and are stored as constantly changing variables that we will designate as LPX (last position-X) and LPY for X and Y respectively.

If the light pen is held at a certain point on the screen, an interrupt will be generated when the beam is picked up by the light pen - this will clearly occur at the X and Y positions pointed to by the light pen. The programmer can then obtain the LPX and LPY positions, and mould these into the program as he wishes.

Now the screen is made up of a large number of raster points - perhaps as many as 1024^2 - and the array element pointed at will take up more than one of these. Some method of referencing a number of raster locations to a particular array element is required. This is handled by assigning a control X,Y location to each character - the control location can then be changed back into the appropriate array position in the main array by using a specially written function. Figure A.3 shows how a group of raster points defining a 'O' which is used to represent a single cell, are referred back to the control location.

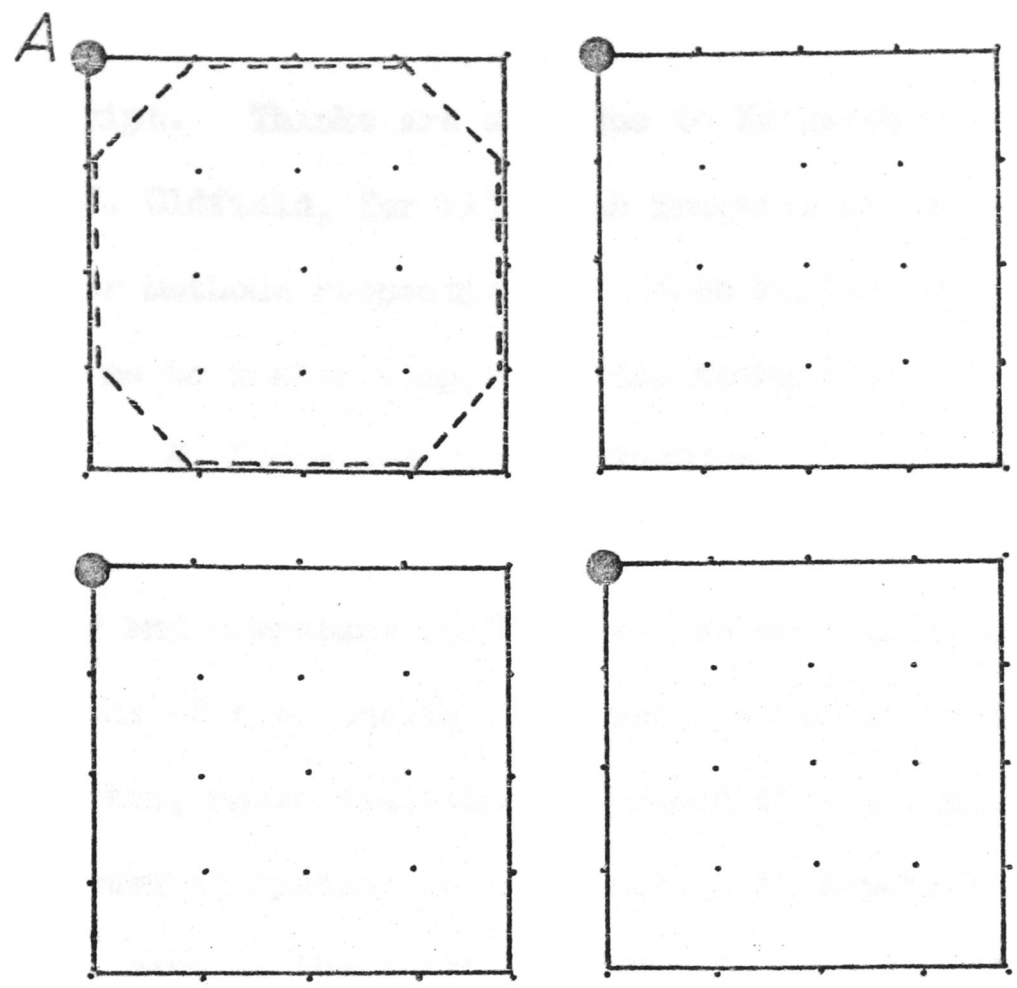


FIGURE A.3 Character representation on the video screen. 100 raster points are shown as dots, and each 5*5 square defines a single 'character area', marked here with solid lines. The top left square holds the character 'O', and the others are empty. Any light pen hit on one of the lines of a character will be recorded as a hit on the 'reference' point of the character, in the case of the 'O' it is marked 'A'here. Each reference point is equivalent to a main array location.

The programmer will have prepared the program to receive the control X and Y co-ordinates, and a variety of steps may be carried out before transferring back to light pen operation. We have already seen some of the sorts of operation that might be used.

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