# PATTERN FORMATION

# AND

# INTERCELLULAR COMMUNICATION

# IN THE ALGA STIGEOCLONIUM

---

A thesis presented for the Degree of Doctor of Philosophy

at the Australian National University

by Lawrence Brian Dooley, July 1980.

---

# CONTENTS

CHAPTER 2 : DEVELOPMENTAL MORPHOLOGY AND LIFE CYCLE OF STIGEOCLONIUM

CHAPTER 3 : EXPERIMENTAL STUDIES OF BRANCHING PATTERNS IN *STIGEOCLONIUM*

CHAPTER 4 : A MODEL OF PATTERN FORMATION

## DECLARATION

I declare that the work presented in this thesis is my own original research and has not been presented for a degree at any other University.

L.B. DOOLEY

July 1980.

## ACKNOWLEDGEMENTS

I am indebted to my supervisors Dr.H.J.Marchant and Professor B.E.S.Gunning for their guidance and support during this study. I wish to thank Professor D.J.Carr, Professor of Development Biology, Research School of Biological Sciences, Australian National University, for his continued encouragement and advice during this study.

Discussions with fellow research students, Allan Browning, Gordon Drake, Noah Pickles-Faud and Robin Scott have often aided this research.

Financial assistance from the following sources is gratefully acknowledged:

Commonwealth Government and the Australian National University for postgraduate scholarships.

The Australia-Japan Foundation and the International Society of Developmental Biologists for travel grants which enabled the presentation of a paper (Dooley, L.B. and Marchant, H.J. Proceedings of the VIII$^{th}$ International Congress of Developmental Biologists) in Tokyo at the VIII$^{th}$ International Congress of Developmental Biologists.

The Ian Potter Foundation and the European Molecular Biology Organisation for travel grants to attend the E.M.B.O. workshop on "Mathematical Models for Biologists" in Israel.

The assistance of Mr. E. Hines with the printing of photographs and Mr. R. Jardine with statistics is gratefully acknowledged.

## ABSTRACT

The developmental morphology and life cycle of Stigeoclonium was studied as a prelude to experimental investigations into the determinants of branching patterns in this alga. A general model based on the descriptive and experimental studies presented in this thesis, and placed in the broader context of plant and animal development, is discussed in the last chapter.

The filamentous green alga Stigeoclonium is among the simplest of branched organisms and grows by intercalary division without producing the complication of age gradients during pattern formation. It consists of a single file of cells. The cells are interconnected by plasmodesmata of approximately 30 nm in diameter.

In an unbranched erect filament of this alga all cells are morphologically equivalent, with the exception of the apical cell. The apical cell has a morphological polarity which none of the other vegetative cells show until they initiate branches. During branch development the morphological equivalent of an apical cell arises from an undifferentiated vegetative cell and later forms a highly differentiated hyaline hair cell. Ultrastructurally branch initiation involves cytoplasmic reorganisation of the nucleate cytoplasm to the site of the branch initiation and an expansion of the vacuole at the opposite end. The first branch in an erect filament is initiated at random but is associated with an asynchronous division of the cell immediately before it initiates the branch.

The branching patterns result from a dynamic equilibrium between transverse cell division and branch initiation. Cell division increases the spacing between branches while branch initiation decreases the number of cells between each branch. The spacing pattern most commonly has six cells between the branches.

Experimental studies on the branching patterns in Stigeoclonium involved a number of physical and drug treatments to investigate the factors controlling branch spacing and polarity.

The expression of branch polarity requires the movement of the nucleus and associated cytoplasm to the site of polar branch initiation. When this movement was prevented by maintaining cells at incipient plasmolysis, apolar branches were produced from the centre of the cell at right angles to the main filament. Altering the position of the nucleus by centrifugation also led to a change in the site of branch initiation. If binucleate cells are produced by caffeine treatment branches arose adjacent to both nuclei. The movement of the nucleus was inhibited by cytochalasin B but not colchicine treatment, suggesting that microfilaments may be involved in the polar migration of the nucleus during branch initiation although no microfilaments were observed in ultrastructural examinations.

Several physical techniques (plasmolysis, cutting, laser microbeam) which disrupted symplastic continuity demonstrated the importance of this pathway for transport from the apical cells to the base of the plant in controlling the position of branches. Each cell within the filament has

the potential to produce a branch and this potential is realised upon temporary isolation from the inhibitory influence of apical cells.

Calcium may play a role in regulating the intercellular transport of a branch inhibitor. The localised deposition of callose near the cross wall areas during plasmolysis required free calcium, and EDAX studies suggested a build up of calcium near the cross wall areas during plasmolysis. Prolonged treatments with the calcium ionophore A 23187 disrupted the normal polar initiation of branches and this could be due to changes of an intracellular calcium gradient associated with the cell cortex.

In the last chapter a model of pattern formation in Stigeoclonium is discussed. The branching patterns in Stigeoclonium could be expressed through a dynamic interplay between the nucleus, the cytoplasm and the plasma-membrane which is determined by the intercellular transport via plasmodesmata of an inhibitor to branching. The data are consistent with a model in which the position of branches is controlled by the intracellular transport of an inhibitor to branching which binds to a plasma-membrane component that controls calcium influx into the cell.

# CHAPTER 1 : THE PROCESS OF BIOLOGICAL PATTERN FORMATION

## 1.1 PATTERN AND FORM

The perception of patterns, both spatial and temporal, have been of paramount importance in the evolution of mankind. The Palaeolithic hunter-gatherers relied on their ability to predict the pattern of animal and plant distribution. With the development of agriculture in the Neolithic age, survival depended increasingly on the recognition of seasonal weather patterns for planting and harvesting crops. Some of the very earliest religions, such as Sun worship, almost certainly were born of this increasing dependence on the seasonal cycles of nature. It is obvious that "the need to recognise patterns arises in most human situations, whenever the brain receives information from any of the senses. The human brain is a pattern recogniser par excellence, providing a continuing challenge to the scientist who can, at the most, suggest systems which are mere shadows of the incredibly powerful entity they seek to replace." (Duff, 1977).

Pattern has played an important part in artistic expression and appreciation. Read (1949) has defined pattern as "some degree of regularity within a limited frame of reference", often perceived in a work of art as "the distribution of line and colour in certain definite repetitions."

By contrast, Read (1949) defines the form of a work of art as "its shape, the arrangement of its parts" which does "not imply regularity, or symmetry, or any kind of proportion." The distinction between pattern and form has also been made in biology. Biological pattern formation is

defined as the spatial control of cellular differentiation while form refers to changes in shape and the forces that generate it (Wolpert,1971).

Much of the beauty of nature is derived from our perception of patterns (Stevens, 1976). Four basic patterns are repeated throughout nature: the spiral; the random meander; the explosion; and various branching patterns. Joining random points in space so that two points connect along only one path with a central point results in variations of these four basic patterns. (Fig.1.1). Space-filling models show that branching patterns combine a shortness as well as a directness of path when linking points in space. The widespread occurrence of branching patterns in such diverse systems as rivers, arteries and trees may be a consequence of their favourable space-filling properties.

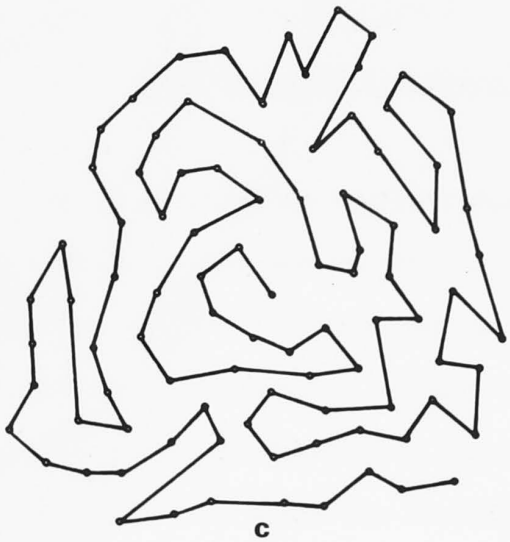
Pattern formation is one of the major unsolved problems in biology. As with many problems in biology, pattern formation can be analysed at a number of levels of organisation. At the phenomenological level the rules governing the development of multicellular organisms can be studied, while at the biochemical level studies on intercellular communication and gene regulation can be undertaken. One of the most intractable problems is the identification of the elusive morphogens which are held to control the development of patterns. However, it is likely, as several authors have maintained (Wolpert, 1971; Cooke, 1975), that an understanding of pattern formation at the phenomenological level is a necessary prerequisite to the success of any attempts to isolate morphogens those chemical or ionic signals that co-ordinate development.

Fig.1.1 The Four Basic Patterns in Nature

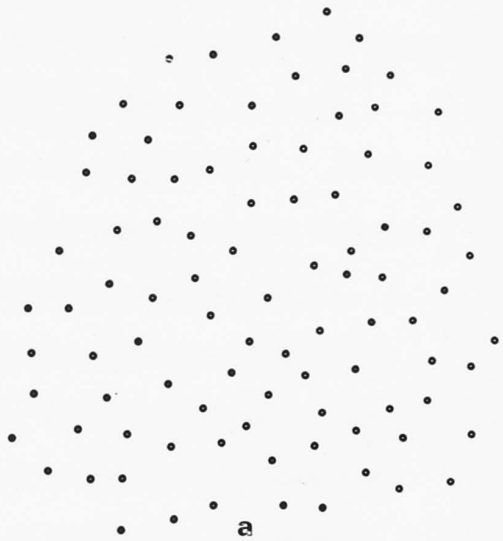
Joining random points in space (a) so that two points connect along only one path with a central point results in variations of four basic patterns: the spiral (b); the random meander (c); the explosion (d); and branching patterns (e).

(From Stevens, 1976).

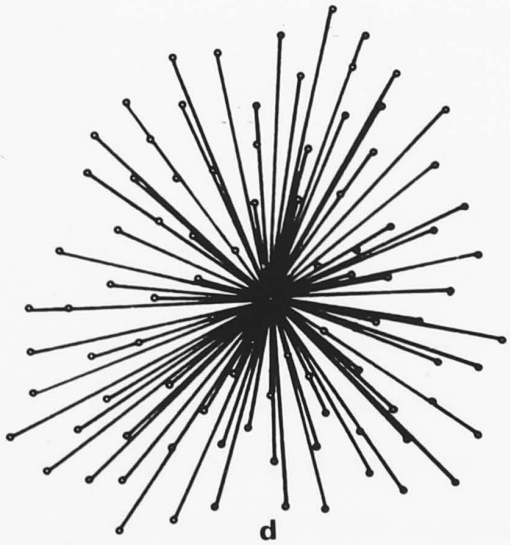
Examples of these four basic patterns for the multicellular development of plants are discussed on p. 210.



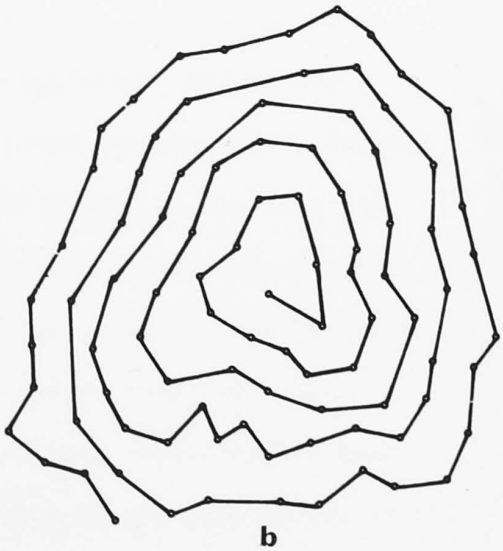
c



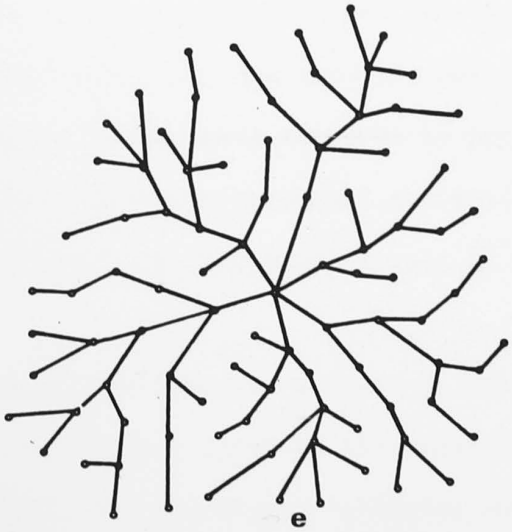
a



d



b



e

## 1.2 THE PROCESS OF BIOLOGICAL PATTERN FORMATION

Pattern formation involves dynamic spatial and temporal interactions that generate and maintain a regular pattern during the development of an organism. As with most complex biological processes, pattern formation can be analysed at various levels of its organisation. Initially pattern formation was studied at the multicellular level and the concept of the developmental field was introduced to explain the regulative features of patterns. The concept of the morphogen was proposed in an attempt to explain the biochemical control and integration within the organism of developmental processes. The major theories that have been proposed involve the intercellular diffusion of morphogens to form a prepattern (Turing, 1952) or developmental gradient (Wolpert, 1971). For example, neurosecretory chemicals which can promote formation of head and tentacles (Schaller, 1973) and suppress formation of buds in Hydra (Berking, 1977) have been isolated. However,the recent finding that nerve-free hydras can undergo normal pattern-regulation (Marcum and Campbell,1978; Campbell, 1979) suggests that these neurosecretory factors are either not essential for the development of Hydra, or at least not the only means by which it can take place. Therefore there is, as yet, no unequivocal evidence that any one chemical can truly be regarded as a morphogen in the sense required by pattern formation theories. Thus although the phenomenological explanation of pattern formation has been successful it has proved difficult to extend this to the biochemical level.

In essence, the process of pattern formation in a developmental field can be considered as the generation of a signal, its intercellular transport, and expression following interpretation by the cells.

In this chapter properties of developmental fields are discussed and various theories that have been proposed to explain the generation of the signal, its transport, and its interpretation are compared.

## 1.3 THE FIELD CONCEPT IN DEVELOPMENTAL BIOLOGY

The field concept in developmental biology arose from Driesch's discovery that spatial position is as important as developmental age in determining the fate of embryonic tissues (Driesch, 1908). He stated that the development of a part is a function of its position in relation to the whole. Child (1915, 1941) regarded the developmental field as a physiological gradient while Dalcq (1938) considered it as two opposite gradients with their ratios specifying position. The concept of "threshold values" of morphogenetic potential was introduced by Dalcq and Pasteels (1937, 1938) to explain the appearance of qualitative differences within a field initially characterized only by a quantitative variation of its morphogenetic potential. It was held that after the threshold values had been established, development would be different either side of the threshold.

The history of the field concept in developmental biology has been surveyed by Nieuwkoop (1967). Summarizing the various concepts, he defined the morphogenetic field as "characterized by a continuous variation of certain properties of the living biological system as a function of position and/or time, leading to an increase in the structural and functional multiplicity of the system." This definition has a dualistic character in that it not only includes the intrinsic properties of the system, but also describes its causal effect within the organism. Biological fields can at present only be detected indirectly from

secondary morphological differentiation: the end-product of a long chain of reactions. Their detection, moreover, requires experimental intervention to distinguish field effects from other strongly interwoven developmental processes (Nieuwkoop, 1967).

Waddington (1966) regarded the developmental field as a topological notion. "Any precise definition of a field must therefore require reference to a multidimensional space, which would have axes on which one could plot not only positions in time and the three dimensions of space but also the concentrations of essential chemical compounds."

At present no precise definition of a field is possible and we are left with the less than satisfactory definitions of Wolpert (1971) : "a field is that region in which all cells are having their positional information specified with respect to the same set of points"; Robertson and Cohen (1972) : a field is "a collection of functionally coupled cells, the development of which is under control of a single system"; and Meinhardt (1978) : "A field can be defined as an area which is controlled by a set of diffusible morphogens."

### 1.3.1 Properties of morphogenetic (developmental) fields

#### 1. Regulative Fields

Regulation refers to the capacity of developmental fields to maintain an invariant pattern over a given size range. Two basic types of regulation have been observed: morphallaxis and epimorphosis.

(a) Morphallaxis

Morphallactic regulation involves remodelling of the missing parts from the residue after cutting. In some planarians, and hydroids such as Tubularia, morphallaxis or rearrangement of existing cells rather than production of new cells at the cut surface results in the regeneration of the whole but diminished organism (Morgan, 1901).

(b) Epimorphosis

In epimorphosis there is growth of tissue to form a blastema in animals and callus in plants which then gives rise to the regenerated regions and produces a pattern in proportion to the whole organism. Epimorphosis has been most clearly demonstrated for amphibian limb regeneration following amputation (Hay, 1966).

In Wolpert's (1969, 1971) theory of positional information duplication in epimorphosis is explained if cells cannot have positional values more proximal than the level from which they were derived but they can have positional values corresponding to more distal regions.

2. Non-regulative or mosaic fields

In mosaic fields, removal of a small piece of the system results in a defect because there is very little interaction between the cells (Wolpert, 1971). The differences that arise between the cells reflects differences already present within the egg. The presence of both mosaic and regulative features in the same system has been illustrated for the

squid and in general, most systems show both regulative and mosaic aspects, but at different times. Some groups, such as insects, display mosaicism and regulation to varying extents. Development is probably always seen to be regulative if the egg can be manipulated early enough (Cooke, 1975).

## 1.4 MODELS OF PATTERN FORMATION IN DEVELOPMENTAL FIELDS

There are two major classes of models to explain biological pattern formation: the prepattern theory and the positional information theory.

In the prepattern theory a spatial singularity of the morphogen is correlated with the site of cytodifferentiation. The weakness of the theory is that it does not readily account for the regulative properties of many developmental fields.

In the positional information theory a cell's position is established by reference to a graded property between the two boundary points of the developmental field. One attribute of positional information theory is that it can readily explain the regulative properties of developmental fields. One defect lies in its dependence on a need to fully understand the mechanism whereby a cell interprets the positional information.

Wolpert's (1971) positional information analysis of pattern formation is presented at the phenomenological level. By contrast, the reaction-diffusion systems of Turing (1952) start at the biochemical level

with biologically plausible reactions that yield a prepattern of spatial and temporal organization.

## 1.4.1 Prepattern theories of biological pattern formation

### Reaction - diffusion systems

### Turing's Reaction-Diffusion Model

In a highly original paper, Turing (1952) suggested that morphogenesis could be explained by a system of interacting chemical substances called morphogens. He proposed that these morphogens formed a prepattern by diffusing through the tissue and reacting together auto- and cross-catalytically. This reaction-diffusion system develops a pattern because random disturbances bring about a breakdown in the inherently unstable homogeneous equilibrium.

The general form of the reaction-diffusion equations for two morphogens u and v can be written as follows (Fife,1977):

$$u_t = D_1 \nabla^2 u + f(u,v) \qquad 1a.$$

$$v_t = D_2 \nabla^2 v + g(u,v) \qquad 1b.$$

$u_t$ and $v_t$ are the concentrations of the two morphogens at time t

$D_1$ and $D_2$ are the diffusion constants

$\nabla^2$ is the three dimensional diffusion term $(\delta/_x{}^2 + \delta/_y{}^2 + \delta/_z{}^2)$

f and g are source terms derived from the assumed reaction schemes, auto-

and cross-catalysis. The source terms f and g describe the reaction kinetics of the two morphogens u and v.

## The Gierer-Meinhardt Model of Pattern Formation

The model of Gierer and Meinhardt (1972) is based on a reaction-diffusion system of the activator-inhibitor type.

Referring to the reaction-diffusion equations 1a. and 1b, u would be an activator and v would be an inhibitor which interact auto- and cross-catalytically. Increasing the amount of u further enhances the production of u and v, whereas increasing v has the opposite effect.* Also the inhibitor diffuses more rapidly than the activator i.e. $D_2 > D_1$.

The analysis of these equations by computer simulation (Gierer and Meinhardt, 1972) has shown the appearance of spatial patterns of various types. This model assumes a prexisting inhomogeneity in the system to explain the generation of polarity and a shallow gradient for one of the components. The stability of the pattern has not been established.

## The Belousov-Zhabotinski Reaction as a model system to study reaction-diffusion kinetics

Biologists have recently become interested in the Belousov-Zhabotinski reaction as a model chemical system exhibiting both temporal and spatial oscillations which are less complicated than biological oscillators.

* This results in a feedback inhibition which is necessary to maintain the long-range inhibition evident in spacing patterns (see Gierer and Meinhardt 1972).

The Belousov-Zhabotinski reaction consists essentially of the oxidation of malonic acid by bromate in the presence of a cerium catalyst which oscillates between two oxidation states, cerous and ceric ions (Atlan, 1975). The reaction oscillates regularly at intervals of about one minute producing travelling concentration waves which are associated with the dramatic changes of oxidation bands. The temporal oscillations involve an autocatalytic reaction while the spatial oscillations are attributed to a reaction-diffusion process (Murray, 1976).

Such processes of auto-catalysis and reaction-diffusion were first suggested as a basis for biological morphogenesis by Turing (1952). Thus it is hoped that a greater understanding of the Belousov-Zhabotinski reaction may help to elucidate far more complex biological oscillations and ultimately provide insights into the process of biological pattern formation.

## Dissipative Structures and Development

Biological organisms are regarded as open systems in a state of dynamic equilibrium consisting of irreversible reactions which maximize free energy and minimize entropy (von Bertalanffy, 1952).

Prigogine commented in 1947 that "classical thermodynamics is an admirable but fragmentary theory. It is fragmentary because it can be applied only to equilibrium states in closed systems. One should therefore attempt to find a more general theory which covers states of disequilibrium as well as equilibrium." (Prigogine, 1947).

Prigogine and his colleagues (see Nicolis and Prigogine, 1977) have extended thermodynamics to irreversible processes and applied their concept of dissipative structures to the origin, evolution and development of living organisms.

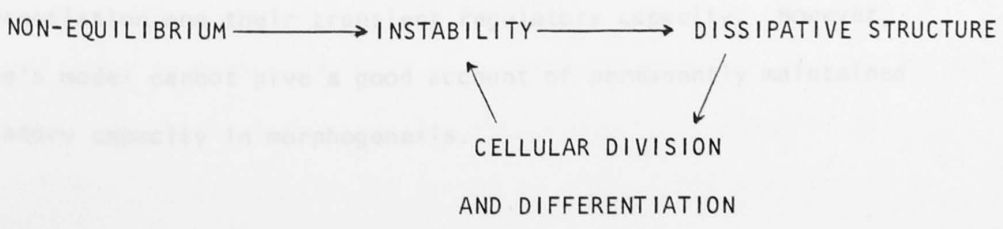
In some systems far from equilibrium a type of order prevails which has been termed "order through fluctuations." Although in a system that is near to equilibrium any fluctuation away from that state will be damped down and equilibrium rapidly restored, there also exists a class of steady state systems, 'dissipative structures' in which, under the right conditions, fluctuations are amplified. These structures can then change from a previous, ordered state to a new ordered state of lower entropy. These systems must be steady-state, open and far from equilibrium; the situation which prevails in biological systems. The notion of a dissipative structure implies both a thermodynamic condition (a critical distance from equilibrium) and a mathematical condition, namely the bifurcation of a new solution arising beyond the instability of the thermodynamic branch (Nicolis, 1975).

Prigogine has shown that dissipative structures can arise in reaction-diffusion systems involving auto-catalysis such as Turing's equations and the Zhabotinsky reaction (Nicolis and Prigogine,1977). The fundamental advantage of this idea over older theories of pattern formation (morphogenetic gradients) lies in the fact that the morphogenetic field is not postulated to be inhomogeneous from the beginning. Rather it explains how inhomogeneity may arise.

Analysis of a reaction-diffusion system involving auto-catalysis has shown that the behaviour of this dissipative structure is simply

related to a parameter $D/L^2$ where D is the diffusion coefficient of the morphogen, L the length of the system (Prigogine and Lefaver, 1975). This parameter measures the coupling between neighbouring spatial regions and has therefore been termed the diffusive coupling. Naturally, the diffusive coupling $D/L^2$ depends strongly on the size of the system. The same biochemical network could give rise to a spatial structure, a propagating wave, or a uniform limit cycle, depending on whether it operates in an isolated cell or in a macroscopic "field of cells" (Nicolis, 1975).

It is proposed that during growth, the response of the organism to distributions of morphogens gradually creates conditions which destabilize the initial pattern. At this point of instability, a new pattern appears which in turn controls the developmental process until itself destabilized by growth. Using computer simulation, Martinez (1972) has analysed the role of cellular division in this succession of instabilities. It was found that the instabilities control their own boundaries, or more exactly the relation between boundary values and the system dimensions via cellular division. Cell division alters the ratio $D/L^2$ which destabilizes the original pattern. This can be summarized as follows:



## Size invariance in reaction diffusion models of dissipative structures

Babloyantz and Hiernaux (1975) have defined the conditions that are necessary for the non-linear systems (such as reaction-diffusion models) to permit size invariance.

If the diffusion gradient of positional information is linear as postulated by Crick (1970) and Wolpert (1971) then size invariance implies that if a system of length L has a concentration m of morphogen at point r and if the size of the system is reduced to L' there must be a point in the new system with the same concentration m as before and such as:

$$r'/L' = r/L$$

For a reaction diffusion system size invariance requires that the diffusion coefficient ($D_m$) also changes so that:-

$$D_m/L^2 = D'_m/L'^2$$

Recent studies of Lacalli and Harrison (1978) have shown a regulative capacity of Turing's model when it is used to explain early stages of pattern formation for slime moulds. Turing's model provides a complete account of the initial establishment of the regions of cell differentiation and their transient regulatory capacity. However, Turing's model cannot give a good account of permanently maintained regulatory capacity in morphogenesis.

## Dissipative Structures and the development of Drosophila

A recent model of pattern formation in Drosophila (Kauffman et al. 1978) provides an elegant synthesis of many strands of thought including gradients, thresholds, a binary combinatorial epigenetic code and dissipative structures.

The development of the adult fly initially involves a regionalization of the embryo into a number of territories which will become the various imaginal disks, and secondly, the subdivision of each of the disks into compartments. Transdetermination of imaginal disks, whereby the state of determination of a disk is changed, has occasionally been observed. Homeotic* mutants can result in the conversion of one compartment to another. It is believed that the compartment boundaries are the thresholds between the on and off states of the wild type alleles of so-called selector genes which undergo the homeotic mutations.

An analysis of transdetermination in the imaginal disks of Drosophila led Kauffman(1969) to the conclusion that the selector genes act like a binary switch capable of being fully on or fully off. The states of determination of the disks are given by a combinatorial 'epigenetic code' or 'on' and 'off' states of the selector genes.

In a more recent model (Kauffman et al. 1978) the switching of the selector genes is controlled by a threshold concentration of a diffusible morphogen. The spatial patterns in the morphogen are obtained by considering a reaction-diffusion system as a dissipative structure so

* Homeotic mutants occur when cells with prospective developmental fates as part of one organ actually differentiate in the form of a completely different organ.

that various pattern modes are generated depending on the size of the system and the diffusivity of the morphogen. As has been discussed before the pattern of spatial organization in a dissipative structure depends critically on the ratio $D/L^2$ with D as the diffusivity and L the length of the system.

The early insect embryo (syncytium stage) does not grow in size, but changes in the diffusivity of the morphogen due to cellularization at the blastoderm stage could initiate the symmetry breaking that is necessary to establish new pattern modes. It is likely that as the syncytium becomes partitioned into cells by cell membranes, resistance to diffusion increases so that effective diffusion constants are made smaller. If diffusion constants become smaller while the ratios of the diffusion constants do not change, the effect is to shorten the unstable wavelength. Mathematically, the effect is the same as if the unstable wavelength remains constant and the spatial domain grows. As the wavelength becomes shorter, a succession of different patterns fit on the egg at a discrete succession of permitted wavelengths. The sequential activation of each binary switch by the successive formation of boundaries creates a binary combinatorial "code word" specifying each terminal compartment.

This model predicts the major features of Drosophila development. The Drosophila egg is elliptical in shape and as a consequence of this the model predicts that longitudinal compartmentation should proceed first, followed by dorsoventral subdivision as the wavelength gradually shortens so that it can fit around the circumference of the egg. Thus the model

predicts the laying down of both axes of the body with the same mechanism and also a sequence of subdivisions which is consistent with the known embryology of Drosophila.

The model predicts size invariance in the sequence and location of compartmental lines for moderate variations in the size of the egg. The size invariance follows from the restrictions on the wavelength imposed by the no-flux boundary conditions - the spatial gradient of concentration at the boundaries must be flat along lines perpendicular to the boundaries. The further condition that there are only a small finite number of binary switches limits the number of chemical patterns that can be recorded and acted upon by the egg. While the nodal compartment lines are size-invariant, they are not shape invariant.

The model also predicts the frequency of particular trans-determinations based on the number of binary switches that must change. The model's assumption that a homeotic mutation acts by altering the states of a binary "selector gene" is consistent with the observed data.

### 1.4.2 The concept of positional information

Wolpert (1971) has formalized the gradient theories of development first proposed by Dalcq (1938) and Child (1941) with the concept of positional information.

A positional value is regarded as a cellular property which is graded from one end of a file of cells to the other between two boundary regions. The positional value can be considered to provide the cells

with a map which allows them to locate their position within the system or unit field of cells; a pattern is formed through the interpretation of the positional value by the particular cells. The concept of positional information is probably best illustrated by Wolpert's French Flag model. Here the problem is to explain how a file of cells can form a pattern such that there is always one-third each of blue, red and white. Wolpert, (1969, 1971) proposed a simple source and sink model of a single gradient of positional information with threshold values specifying the differentiation of the cells into blue, white or red. More complex models providing positional information could involve two intersecting gradients with the positional value determined by the ratio between them.

In terms of positional information, pattern formation is a two step process: first, the specification of positional information within the field, and secondly, the interpretation of this information by the cells, resulting in the expression of the pattern.

An important feature of Wolpert's theory is that the same positional information may be used to provide very different patterns, since variations of pattern mainly depend not on variant spatial distribution of the positional information but on variant interpretation of it. This fact distinguishes Wolpert's positional information theory from earlier prepattern theories. The prepattern theory requires some correspondence between the variation in the value of a parameter such as a morphogen and the observed pattern. In the prepattern theory, a spatial singularity in the concentration of a morphogen is correlated with the position of cytodifferentiation.

One of the most successful aspects of Wolpert's theory is its explanation of the regulative and size-invariant properties of developmental fields. The property of size-invariance depends on the maintenance of the boundary values of morphogen concentrations and the threshold concentrations for differentiation over varying distances. Therefore a smaller field will have a steeper gradient of morphogen but since the cells have the same threshold for differentiation a smaller pattern with exactly the same proportions will develop. In essence, the boundary values are assumed to be fixed by a mechanism which is independent of the length of the axis so that a regulating gradient results. Morphallactic and epimorphic regulation in developmental fields is explained by the conservation of boundary values for the morphogen. In morphallaxis the establishment of an identical boundary value for the morphogen at the cut surface and the maintenance of a new gradient ensure size-invariance of the pattern.

The success of Wolpert's model in explaining the phenomenological aspects of development has not been extended to other levels of analysis. The distinction between positional information and its interpretation makes universality of developmental principles possible. Different species could employ different codes for interpreting similar gradients of positional information. Thus the burden for creating patterns from monotonically changing spatial variables is then placed upon the cellular interpretative machinery. The positional information theory is in danger of simply transferring the problem of pattern formation "from one area to another" (Goodwin, 1976).

### The phase-shift model

Goodwin and Cohen (1969) have proposed a phase-shift model for the spatial and temporal organization of developing organisms. They propose that a map of cell position arises from wave-like propagation of activity from localized cells acting as clocks or pacemakers. The gradient of positional value is established by the propagation of two waves with different velocities from a boundary pacemaker cell, the phase difference between the two waves providing a gradient in positional information.

The model is mathematically quite complex but a summary of its potential value is illustrated in the original paper for regulating somite numbers in the vertebrate embryo. A series of similar structures might form at regular intervals along the axis where maxima of the two periodic signals coincided. A simple analogy is that of two sound waves of different frequencies which produce "beats" when their maxima coincide, at regular time intervals. Alterations in the frequency of the two signals could regulate the pattern within different tissue sizes.

The strength of the model is that it introduces the interaction between space and time in development. The temporal biochemical organization of an individual cell is converted by functional coupling between cells into a spatial ordering of the temporal organization.

## 1.5 INTERCELLULAR COMMUNICATION DURING FIELD ESTABLISHMENT AND REGULATION

Intercellular communication during development may involve either

of two fundamental processes: random diffusion or a directed signalling mechanism. Crick (1970, 1971) has discussed the implications of both these mechanisms.

A linear gradient of morphogen can be established by diffusion if a dynamic equilibrium is maintained between a source and a sink for the morphogen. Diffusion is a random walk process and the dimensions of the diffusion constant are $L^2T^{-1}$ (L is length, T is time).

The mean distance of diffusion ($\bar{x}$) in time t can be described by:

$$\frac{\bar{x}^2}{t} = \text{constant}$$

Thus diffusion processes are rapid over short distances but very slow over large distances.

By contrast, a simple signalling process could be propagated at constant velocity (dimensions $LT^{-1}$) such that:

$$\frac{x}{t} = \text{constant}$$

In this case the difference in time for short and long distance effects is not as great as for a diffusional process.

Crick (1971) has divided signalling mechanisms into two broad categories: amplitude decay and phase difference.

For the amplitude decay mechanism the amplitude of the signal could be fixed at the source and decrease as the signal is propagated. If the

output of each cell was proportional to the input a cell could use the amplitude of the signal to give positional information.

The second type of signalling mechanism relies on the phase difference between two signals as proposed by Goodwin and Cohen (1969). The signals are synchronized at the source but travel with different velocities so that a cell can obtain positional information from the phase difference between the two signals.

Based on considerations of the size of embryonic fields and the time needed to set up a gradient, Crick (1970, 1971) has maintained that diffusion can account for the morphogenetic gradients in embryonic development. "Nature usually has such difficulty evolving elaborate biochemical mechanisms (for example, those used in protein synthesis) that the underlying processes are often rather simple " (Crick, 1970).

The plausibility of diffusional gradients for distances comparable to embryonic fields has been established by Michalke (1977) using monolayers of cultured cells. By culturing wild type rat liver epithelial cells immediately adjacent to mutant cells which cannot incorporate radioactive hypoxanthine into nucleic acids by themselves, gradients of hypoxanthine-derived radioactivity have been observed in the mutant cells. The gradient of radioactivity can be described by an exponential function of the form $e^{-kD}$, where D is the distance from the monolayer of wild-type cells and k describes the slope of the gradient. The concentration of radioactivity decreases by a factor of about 100 over a distance of 1 mm. The gradient is presumed to depend on intercellular diffusion, since this type of metabolic co-operation cannot be detected in cells in which the

communicative gap junctions are defective. Over certain concentration ranges an exponential concentration gradient of diffusible ligand can be converted to a linear gradient by assuming binding of the ligand to a macromolecular receptor which cannot diffuse from cell to cell (Michalke, 1977).

## 1.6 GENE REGULATION: THE INTERPRETATION OF A POSITIONAL SIGNAL

Development involves the sequential and regulated expression of inherited capacities. Both the Operon model of gene regulation in bacteria (Jacob and Monod, 1961) and the Britten-Davidson theory of gene regulation in higher organisms (Britten and Davidson, 1969) involve control of transcription during cell differentiation. Kauffman(1969) has considered the gene to be a binary switch either fully on or off and has developed this to form complex networks of switches which could control the development of the organism.

Pattern formation requires a mechanism for translating the positional signal into a regulation of gene expression which results in cell differentiation at a particular position. The process of cellular differentiation can be regarded as a dynamic interplay between the nucleus, the cytoplasm and the plasma-membrane. It is possible that the plasma-membrane may act as a central regulatory control point by transduction of signals. A model of cell differentiation involving interaction between plasma-membrane and genome has recently been developed by Brunner (1977). While the essential steps involved in the flow of information from the nucleus to cell membrane are well known (transcription and translation) knowledge about the flow of information in the reverse

direction (membrane to nucleus) is scarce. It is believed that signal molecules are bound to the plasma-membrane by receptor molecules and initiate a sequence of reactions in the cell which finally lead to the expression of previously untranscribed parts of the genome.

The evidence for such a mechanism is well established for mitogens, hormones, some enzyme inhibitors, and ions such as calcium (Brunner,1977). Some advantages of regarding the membrane as a transducer of signals to the nucleus are that mosaic development could be explained by an unequal and fixed distribution of receptors at the egg cell surface. Also, development which proceeds as a strict cell lineage could be a function of sequential receptor expression. This model would require some control of membrane receptor mobility and distribution, most probably by the membrane-associated microtubules and microfilaments (Nicolson et al, 1977).

The crucial test of this promising theory of cell differentiation as resulting from an interaction between the plasma-membrane and nucleus has been suggested by Brunner (1972). The experiment would involve the incorporation of membrane areas bearing differentiation receptors for cell type A into intact cells of another lineage (cell type B). An attempt would then be made to transform cell type B into cell type A after stimulation of the type A differentiation receptors by species of triggering molecule appropriate to them.

## 1.7 PLANT DEVELOPMENT

The majority of models discussed in the previous sections are

based on studies with animal systems. It is ironical that although Turing saw some applications of his important reaction-diffusion model to phyllotactic patterns in plants, very few detailed models of pattern formation for plants have been considered until relatively recently. This is possibly because studies on pattern formation in plants have largely been concerned with the description of histology in terms of planes of cell division and cell lineages. Little experimental work has been done on the underlying critical controls of pattern formation in plants. This is despite the fact that plants present certain features which make them very favourable for studies on pattern formation.

In a classical treatise on plant morphogenesis, Sinnott (1960) drew attention to a number of the advantages of studies of the developmental biology of plants. By their indeterminate growth, root and shoot meristems provide regions of prolonged embryonic development as compared with the short period of embryogenesis in animals. The fact that plants often have multiple meristems means that experiments can be undertaken on material of identical genetic constitution.

Except for special cases such as the egg of the marine alga Fucus (see Quatrano, 1978) very little work has been done on pattern formation of plant embryos. This is largely because higher plant embryos are relatively inaccessible to experimental manipulation by virtue of being surrounded by layers of maternal tissue. It is possible that application of the techniques of tissue culture may alleviate the constraints on research on pattern formation in plant embryos.

The sessile plant and particularly its constituent organs such as

the leaves, flowers and fruits may suffer developmental changes in response to changes in the environment, unlike animals which respond to environmental change preferentially, by movement and modification of behaviour. Thus plants present many excellent opportunities for studying important interactions between environment and development.

Plant structure is not complicated by the interactions of diverse tissues such as the nervous system in animal development. The developmental complications introduced by the possession of a nervous system in even a simple animal such as Hydra (Campbell, 1979) have already been mentioned in section 1.2.

In general, plants possess far greater capacity for repair than animals. A single cell isolated from an adult plant may reconstitute the whole plant under appropriate conditions in cluture (Melchers, 1965). Regeneration in animals usually involves replacement of only the lost part while regeneration in plants can often involve the formation of a whole new plant from the excised parts.

That the plant cell is encased by a wall presents certain advantages as well as some disadvantages for studies of pattern formation. For the researcher, the disadvantage of the cell wall is that it prevents ready access to the cell membrane during development. This is partly alleviated by the use of freeze etch techniques with the electron microscope. On the other hand, the cell wall largely eliminates the complication of cellular movement during pattern formation, and it provides a record of the process of pattern formation. Plant protoplasts, cells freed from the cell wall, offer further scope for studies on re-

generation and pattern formation.

Examples of studies on plant development which illustrate some of the advantages of plant systems for studying pattern formation are discussed below.

## The Root Meristem

The prolonged embryonic activity of the root meristem with its precise and predictable patterns of development has been elegantly exploited in studies on the development of the root of the water fern, Azolla (Gunning et al.,1978 a and b). The precision with which the single apical cell divides to produce cell lineages allows the construction of a map predicting the temporal and spatial development of constituent cells in the Azolla root (Gunning et al.,1978a). This precise patterning was used to advantage in studies on the anticipation of the planes of cell division in the Azolla root by pre-prophase microtubules (Gunning et al., 1978b). However the pre-prophase band cannot itself determine cell polarity or the plane of division (Gunning et al.,1978b) and the fundamental problem is the underlying mechanism whereby the pre-prophase bands are positioned. It is possible that this is provided by a gradient of positional information maintained by intercellular communication.

The meristems of root and shoot have clear similarities to the positional fields of animal embryogenesis. The primary meristematic regions of the plant are similar in size to the embryonic fields of animal systems, i.e., 1 mm in width and consisting of 50 to 100 cells (Holder, 1979). As Barlow (1976) said, "The root and shoot meristems

show some features characteristic of a positional field, particularly if we think of the founder cell population as a boundary region." The growth and development of the root meristem could be explained by the interaction of two gradients which provide positional information. Barlow (1976) suggested that one gradient might be provided by cytokinin which would move away from its source in the quiescent centre while the other gradient could be of an auxin which moves towards the quiescent centre with a source in the mature cells of the root apex. The ratio of the cytokinin and auxin could determine the pathway of cell differentiation at any position along the root meristem. Thus it should be possible to develop models of positional differentiation for the plant meristems which are amenable to experimental investigation.

## Vascular Patterns

Vascular tissue in plants is organised in a structurally relatively simple pattern which regenerates well after experimental interventions such as wounding.

Jacobs (1952) clearly demonstrated IAA (βindolyl acetic acid) to be a necessary factor in the regeneration of vascular bundles after wounding in Coleus. Removal of a young leaf above a wound inhibited xylem regeneration but the inhibition could be overcome by applying IAA to the petiole of the excised leaf. Studies on the vascularisation of callus tissue (Wetmore and Rier, 1963; Jeffs and Northcote, 1967) have shown induction of vascular nodules at a particular concentration of auxin and sucrose. Of fifteen sugars tested by Jeffs and Northcote (1967), only sucrose, trehalose and maltose could induce vascular

nodules. These sugars all have an $\alpha$- glucosyl residue at the non-reducing end which might imply some specificity of interaction between this residue and a binding site.

A gradient induction hypothesis which is consistent with all experimental results on the positioning of regenerating vascular cambia after the wounding and grafting of dicotyledonous stems has been proposed by Warren Wilson and Warren Wilson (1961). The essential features of the hypothesis are a gradient of morphogen to the exposed surface which controls the position of regenerating vascular cambium by a threshold mechanism. To account for the tendency of the regenerating cambia to unite with established cambia it is supposed that after tissues have differentiated the morphogen concentration becomes fixed in them and they induce similar levels in the immediately adjacent tissues. In a recent extension of the gradient induction hypothesis, Warren Wilson (1978) has proposed that there are two morphogens, auxin and sucrose, which provide gradients such that the ratio between sucrose and auxin determines the position of vascular differentiation. The plausibility of the theory has been demonstrated by simulations which were found to be consistent with the results from a number of wounding and grafting experiments.

These examples of studies on pattern formation in plants illustrate the advantages of studying the developmental biology of plants and show the possibility of applying models of positional information to plant development. In the next section one of the simplest branched plants is introduced as a geometrically simple system for the study of pattern formation.

## 1.8 THE ALGA STIGEOCLONIUM AS A DEVELOPMENTAL SYSTEM

The complexity of the interactions of processes during the development of an organism makes geometrically simple systems attractive in the study of pattern formation. In the investigation of major problems in developmental biology it is desirable to choose an organism which illustrates a general problem rather than an idiosyncrasy of a particular species. Much of the traditional work on pattern formation has been undertaken on complex animal embryos undergoing development in three dimensions.

Certain plant systems provide advantages due to their geometrical simplicity and an absence of cell movement during pattern formation because of the physical constraints of the cell wall. The branching patterns they show are among the commonest patterns in nature.

Stigeoclonium is among the simplest of branched organisms, excluding the false branching of some procaryotic blue-green algae. The growth of this alga by intercalary cell division means that age gradients are not set up in the organism. Thus, the complications of differences in cell age during pattern formation do not arise. In an unbranched erect filament of Stigeoclonium all cells are morphologically equivalent with the exception of the apical cell. During the development of a branch, the equivalent of an apical cell arises from a normal vegetative cell. Other cells of the branch differ from those of the main filament only in their orientation with respect to that filament. The basal cell from which the branch arises becomes thick-walled and

slightly barrel-shaped. In the final stages of differentiation of a branch, an elongate hair cell is produced at the apex. Stigeoclonium can be considered as exhibiting a relatively simplified example of pattern formation which involves spatial control of cellular differentiation. Details of the developmental morphology and life cycle of this alga will be discussed in the following chapter, experimental manipulations in Chapter 3 and a general model for pattern formation in the final chapter.

The polarity of branching was defined in relation to the site of branch initiation within an individual cell. "Apical" means originating at the apical end of an individual cell, an "apolar" branch was initiated from the centre of the cell, while "basal" denotes a branch originating from the base of a cell. In the majority of cases "apical" branches subsequently grew in the direction of the apex of the multicellular filament while "basal" branches subsequently grew in the direction of the base of the filament. "Apolar" branches usually grew at right angles to the multicellular filament.

## CHAPTER 2 : DEVELOPMENTAL MORPHOLOGY AND LIFE CYCLE OF STIGEOCLONIUM

## 2.1 INTRODUCTION

Stigeoclonium Kützing (order, Ulotrichales; family, Chaetophoraceae) is a filamentous branched Chlorophyte having cells of the main axis and branches of a similar size (Cox and Bold, 1966). Stigeoclonium is a ubiquitous alga able to grow in freshwater environments throughout the world despite the fact that they vary widely in temperature and nutrient composition (Islam, 1963). It is commonly attached to rocks in flowing streams but also grows profusely in still water. Stigeoclonium (L. stigens, sharp + L. clonium, branch) has a heterotrichous growth habit with a prostrate portion that secures the plant to the substrate and from which branched, erect filaments develop. (Fig. 2·1)

## 2.2 GENERAL METHODS AND MATERIALS

### 2.2.1 Source of Stigeoclonium isolates

The culture used throughout this study was isolated from the Stepping-Stones area of Sullivan's Creek on the campus of the Australian National University. No attempt was made to identify the species of the plant because I consider that the criteria proposed for species identification in the genus Stigeoclonium have not been sufficiently established (Islam, 1963; Cox and Bold, 1966). The culture used throughout this study was established from a single zoospore isolate of a single plant from Sullivan's Creek. The culture was maintained as a uni-algal culture with no other algal contaminants by transferring small segments of the upright filaments to fresh culture medium.

Fig. 2.1 A simple, branched, erect filament of *Stigeoclonium*.
Phase Contrast X450.

## 2.2.2 Culture Conditions

### (a) Medium

A modified Pocock's (1960) medium was used. Full details of the medium are given in Table 2.1. Although the medium was undefined in that it contained soil-water, the soil came from the same source and batches of soil-water were prepared in the same way. This medium was used because it gave growth rates and morphologies similar to that observed in nature and *Stigeoclonium* grown in it appeared better preserved ultrastructurally than when other media were used.

Occasionally a fully-defined Bold's basal medium (Nichols and Bold, 1965) was used.

### (b) Lighting

Cultures were grown under a regime of 8 hours dark (0900 - 1700 hrs) 16 hours light (1700-0900 hrs). Illumination was by two 20-watt "daylight" fluorescent lamps. Cultures were grown in 9 cm plastic Petri dishes, placed on shelves approximately 1 foot under the lights and in plastic trays lined with silver foil, giving a light intensity of approximately 2,500 lux at the level of the petri dishes.

### (c) Temperature

The temperature of the growth room was maintained at $20^{o} \pm 1^{o}$ C.

TABLE 2.1a POCOCK'S MEDIUM

| Compound | Stock Solns. (g/100 ml.) | Standard (ml./l) | Modified (ml./l) |
|---|---|---|---|
| $KNO_3$ | 5 | .33 | .33 |
| $MgSO_4$ | 5 | .33 | .33 |
| $K_2HPO_4$ | 5 | .33 | .33 |
| $K_2CO_3$ | 7 | .33 | .33 |
| $CaNO_3$ | 20 | .33 | .33 |
| $FeCl_3$ | 1 | .33 | .20 |
| Soil Decoction | See below | 100 | 100 |
| Hill's Trace Element Solution (see next page) | | | 15 |
| $B_{12}$ soln. | 1.0 µg/ml. | | 2.5 |
| Thiamin | | | .0001 gm |
| Biotin | | | .0001 gm |

Check pH 6.7-7.0

Add vitamins after soln. has been boiled.

Soil decoction:

The soil decoction was prepared by heating approximately equal volumes of soil, rich in organic matter, and tap water in a boiling water bath for about an hour. The mixture was left to cool and settle overnight.

The supernatant was decanted and filtered.

TABLE 2.1b HILL'S TRACE ELEMENT SOLUTION

| Compound | Stock Soln. (g/100 ml.) | Element |
|---|---|---|
| $Na_2B_4O_7.10H_2O$ | 4.77 | B |
| $CuSO_4.5H_2O$ | 0.25 | Cu |
| $CoCl_2.6H_2O$ | 0.238 | Co |
| $MnCl_2.4H_2O$ | 0.198 | Mn |
| $ZnSO_4.7H_2O$ | 0.287 | Zn |
| $(NH_4)_6Mo_7O_{24}.9H_2O$ | 0.176 | Mo |
| $FeSO_4.7H_2O$ | 0.278 | Fe |
| $N(CH_2OOOH)_3$ | 1.91 | NTA (Nitrilo triacetic acid) |

Adjust to pH 2 with 10N $H_2SO_4$

### 2.2.3 Cultures

Unless otherwise stated, young actively growing plants 8-10 days old were used throughout this study.

### 2.2.4 Standard fixation

Stigeoclonium was fixed for one hour at room temperature in one percent glutaraldehyde in Pocock's Medium; washed three times in fresh medium and post-fixed in one percent osmium tetroxide in Pocock's Medium for 1 hour.

### 2.2.5 Standard dehydration

Fixed material was dehydrated in glass Petri dishes on ice by drop-wise addition of a series of graded acetone concentrations over a 2-day period.

### 2.2.6 Standard embedding

Spurr's resin (Spurr, 1969) was used for flat embedding between glass microscope slides, coated with Teflon (PTFE) (Chang 1971).

### 2.2.7 Electron & Light Microscopy

Sections for transmission electron microscopy were cut using glass knives on a Reichert microtome and mounted on Formvar coated copper grids. The sections were stained with uranyl acetate and lead citrate

(Reynolds, 1963) and examined with a Hitachi H-500 electron microscope.

For scanning electron microscopy, material was processed as described by Marchant (1973), critical-point dried and coated with gold-palladium.

The light micrographs were taken using a Zeiss photomicroscope and Kodak Panatomic-X Fine Grain film developed with Kodak D-76 developer.

### 2.2.8 Cell Wall Digestion

Cell walls of Stigeoclonium were enzymatically digested using Cellulysin and Macerase as described by Marchant and Fowke (1977).

### 2.2.9 Cell Size Analysis

The length and width of 1000 vegetative cells was measured in actively growing cultures 8-10 days old during the dark cycle.

The measurements were made to the nearest 2 µm from random microscope fields using a calibrated eyepiece micrometer.

### 2.2.10 Cell Division in Stigeoclonium

The optical brightener Calcofluor White M2R (American Cyanamid Co.) (Hughes and McCully, 1975) was used to stain the cell walls.

8-day old cultures growing on sterile cover-slips in Petri dishes were used for these experiments. These were young actively growing plants. Cultures were stained in 0.1% Calcofluor in growth medium for 10 minutes under green fluorescent lights in the growth room. They were then washed in two changes of fresh growth medium and placed back on the culture shelves. Slides were made at 3 hour intervals in the growth room and were examined with a Zeiss fluorescence microscope using the filters described on p. 112. The culture was restained with Calcofluor after each sampling period. The number of recently divided cells was determined by recording 200 cells from random microscope fields.

2.2.11 Ontogenetic Study of Branch Development

Cells were stained with Calcofluor during the dark cycle (1500 hrs) as detailed above and examined with a Zeiss fluorescence microscope 24 hours after staining. Cell division and branch development were recorded from random microscope fields.

Young filaments which had not branched and were growing on cover-slips were stained with Calcofluor as above. They were examined every hour and the number of cells between the apical cell and the first branch recorded as well as the synchrony of cell division in the filament.

2.2.12 Branching Patterns

Actively growing cultures 8-10 days old were examined and the number of cells between branches was recorded 1000 times from random microscope fields.

Branching patterns for Stigeoclonium freshly collected from nature were recorded as above from the same site as the cultures used throughout this study.

### 2.2.13 Statistical analysis

In many cases the results were obvious and did not require statistical analysis. In other cases Chi-square tests were used to establish the significance of the results and the Chi-square values are given in the particular tables.

The branching pattern for Stigeoclonium in culture was analysed by computer using the Rothamsted Maximum Likelihood Programme (MLP) (Ross 1980).

## 2.3 RESULTS

### 2.3.1 Morphology of Stigeoclonium

The heterotrichous growth habit of Stigeoclonium is well illustrated by Fig. 2.2.

#### (a) Morphology of the basal portion

The prostrate portion develops following settling and attachment of a zoospore and enlarges by apical growth of its rounded, thick walled cells (Fig. 2.3). The degree of development of the prostrate portion is variable (Fig. 2.3 e + f) and some authors have shown a strong dependence of it on culture conditions. Fritsch (1935) observed that the degree of development of the erect system was usually in inverse ratio to the development of the prostrate system. Cox and Bold (1966) found that the proportional development of the basal and erect portions differed depending on culture conditions such as pH, aeration and nutrient level.

Portions of detached erect filament can often re-attach to the substratum by means of rhizoids (Fig. 2.4). Cox and Bold (1966) found that those species of Stigeoclonium in which the basal system was poorly developed or entirely lacking usually produced rhizoids from the lower cells of the erect filaments which presumably aid the attachment of the plant to the substratum. The rhizoids often showed a tortuous or helical morphology. Both the normal basal portion and rhizoids

Fig. 2.2 Heterotrichous growth habit of Stigeoclonium

(a) Scanning electron micrograph showing basal portion (arrow) and erect filaments (arrowhead) projecting out of the plane of the picture. Note the detritus. X650.

(b) Section of basal portion showing layer of material (most likely mucilaginous) (arrow) around the foot cells. X6,500.


a


b

Fig. 2.3 Development of the prostrate, basal portion in Stigeoclonium.

(a) A settled zoospore which has retracted its flagella and is beginning to germinate. Nomarski X850.

(b) Elongate germlings which have undergone a first division. The septa are arrowed. Nomarski X1,200.

(c) The first branch arising from a 3-cell germling. Nomarski X1,200.

(d), (e) & (f).

Development of the basal holdfast in an intricate branching pattern.

(d) Phase Contrast X250, (e) and (f) Phase Contrast X350.

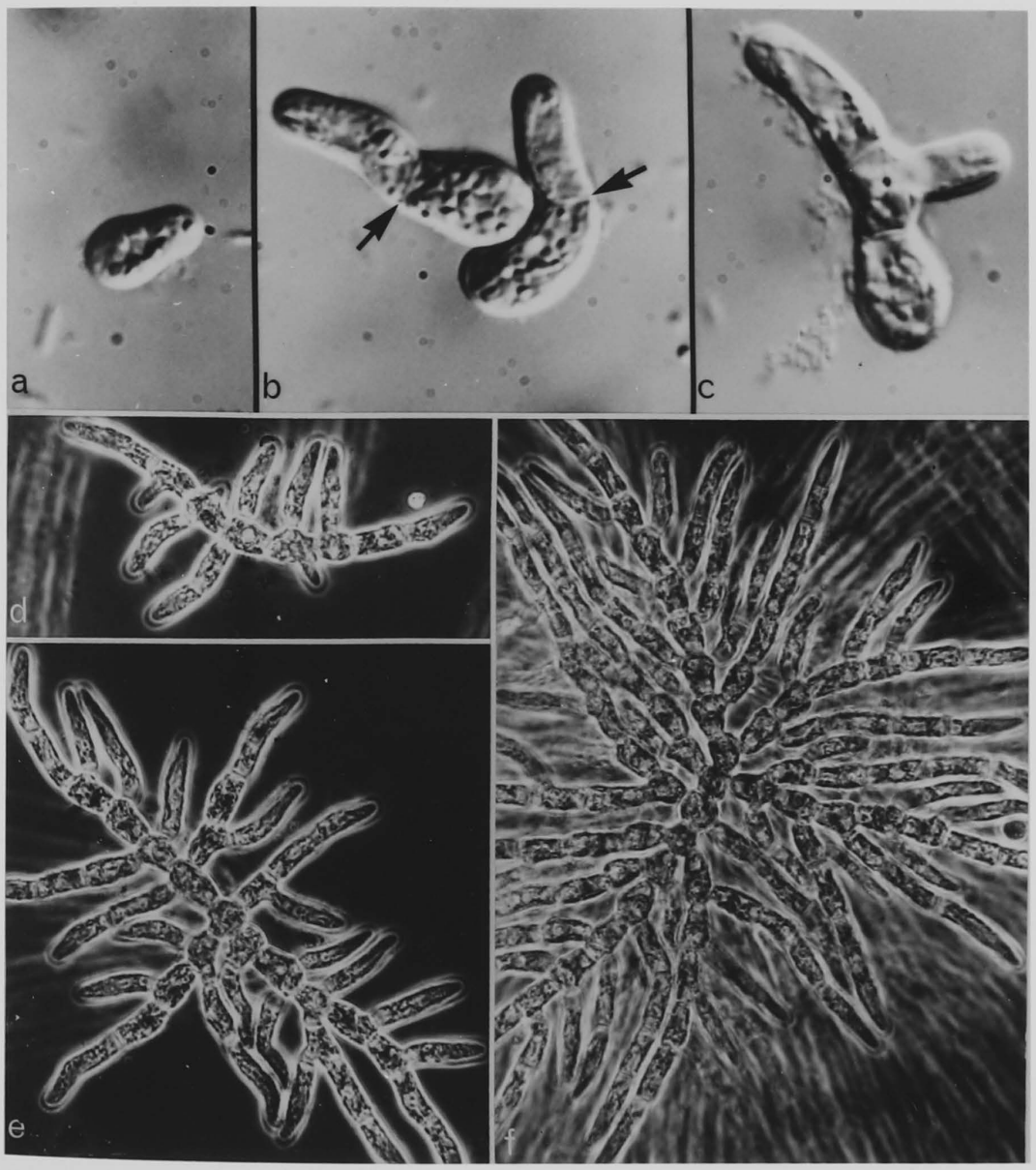
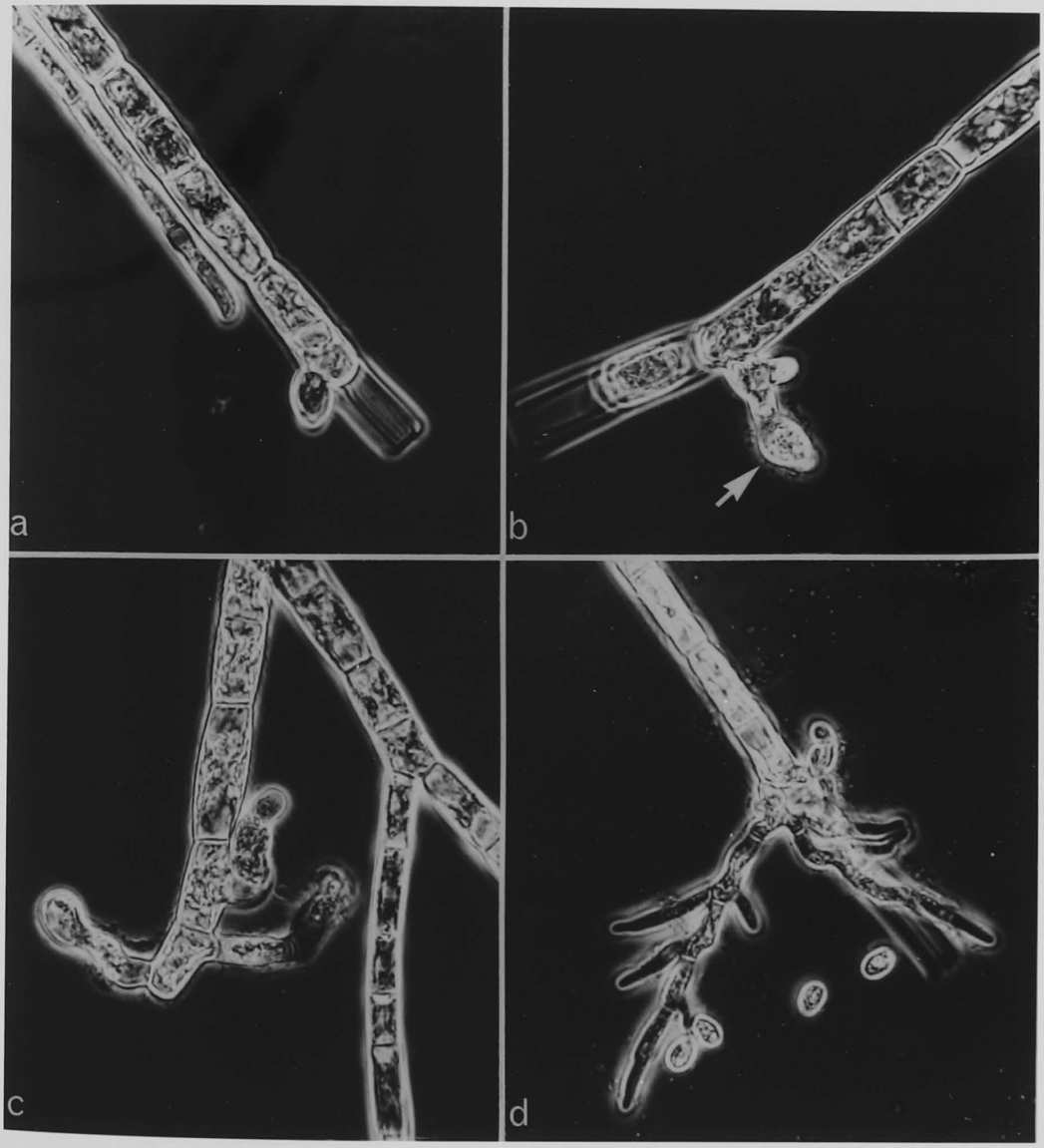

a
b
c
d
e
f

Fig. 2.4 Rhizoid Formation

(a) & (b) Rhizoid formation 24 hours after cutting of a filament. Note material around rhizoid in (b) (arrow) which is probably detritus trapped in mucilage. Phase Contrast X600.

(c) Rhizoid formation 48 hours after cutting. Phase Contrast X600.

(d) Rhizoid formation 72 hours after cutting. Phase Contrast X500.


a
b
c
d

were surrounded by a mucilaginous substance in which detritus became embedded (Fig. 2.2b and 2.4b).

The mucilage is presumably responsible for anchoring the alga to its substratum.

In laboratory culture the basal portion of each plant normally develops on the bottom of the Petri dish after a zoospore settles. Occasionally the basal portion was observed to develop at the air-water interface and the erect portion grew down into the medium. If there were scratches on the bottom of the Petri dish, zoospores apparently preferred to attach themselves to these roughened areas.

(b) Morphology of the erect portion

The erect portion of the plant develops from the basal portion by intercalary or diffuse growth. Lateral branches are produced from undifferentiated cells of the main axis. The erect filaments develop at a random orientation to the basal cells and more than one erect filament can develop from a single basal cell (Fig. 2.5).

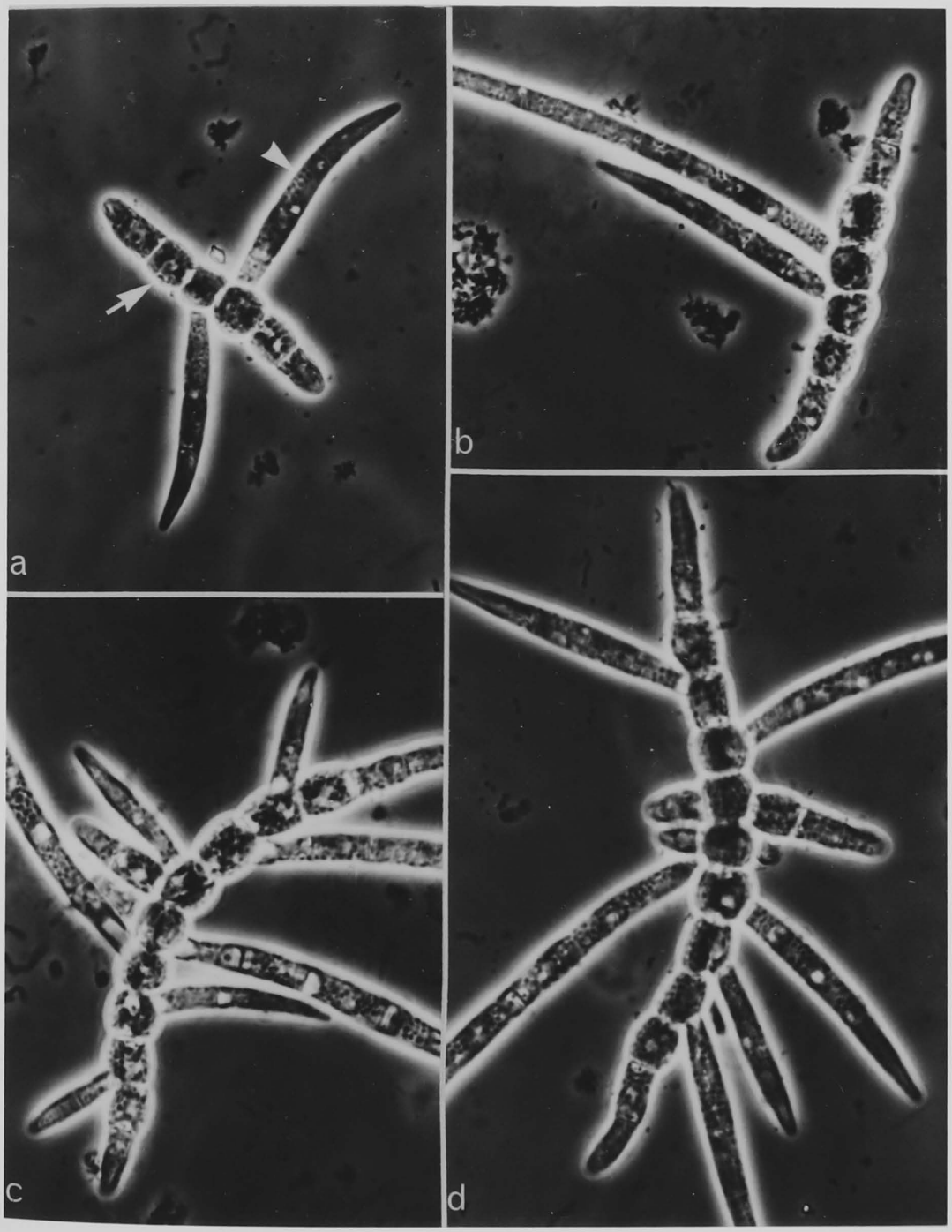
The cells of the main axis and lateral branches are of a similar size; both primary and secondary branches are formed in mature cultures.

Cells of the main axis from which branches arise often become thick-walled and slightly barrel-shaped. As the branches mature, they each develop a terminal hair cell which apparently lacks plastids as observed using light microscopy with Nomarski and phase contrast optics (see Fig. 2.18f). Electron microscopy would be needed to unequivocally establish the absence of plastids.

Fig. 2.5 Development of erect filaments from a reduced basal holdfast.

(a) and (b) The erect filaments (arrowhead) develop at a random orientation to the basal cells (arrow). Both Phase Contrast X350.

(c) and (d) More than one erect filament can develop from a single basal cell. Both Phase Contrast X450.


a
b
c
d

Hair cells usually develop in cultures older than 14 days, consistent with Godward's (1942) observation on Stigeoclonium amoenum that hair production occurred after the end of vegetative growth.

## (c) Organization of Stigeoclonium Cells

The ultrastructure of Stigeoclonium cells has been described in part by Floyd et al. (1972). It is surprising that a common alga such as Stigeoclonium, which exhibits differentiated cells should not have been studied in detail by electron microscopy.

The single nucleus is located in a central zone of cytoplasm between two vacuoles (or vacuole lobes). (Fig. 2.6a). Each cell contains a single large parietal chloroplast (Figs. 2.6b and 2.7a) in which tubules were sometimes observed (Fig. 2.7b).

Two large vacuoles (or vacuole lobes) were located at either end of the cell (Fig. 2.6a) but became reduced in volume as the cells aged (Fig. 2.7a). Older cells had a denser cytoplasm and increased starch deposits (Fig. 2.7a).

During branch development the nucleus moves to one end of the cell. These migrating nuclei often contained densely staining bodies (Fig. 2.8a) and a very fine fibrillar matrix (Fig. 2.8c). Nuclear pores were also observed in sections grazing the nuclear envelope of migrating nuclei (Fig. 2.8b).

Fig. 2.6 General Ultrastructure of a vegetative cell of Stigeoclonium.

(a) Longitudinal section showing terminal vacuoles (v) at either end of the cell, centrally located nucleus (n), parietal chloroplast (ch), pyrenoids (p) surrounded by starch bodies (s) and mitochondria (m). X8,500.

(b) Transverse section through the centre of the cell, showing parietal chloroplast (ch) encircling the nucleus (n). (nc, nucleolus). X20,250.

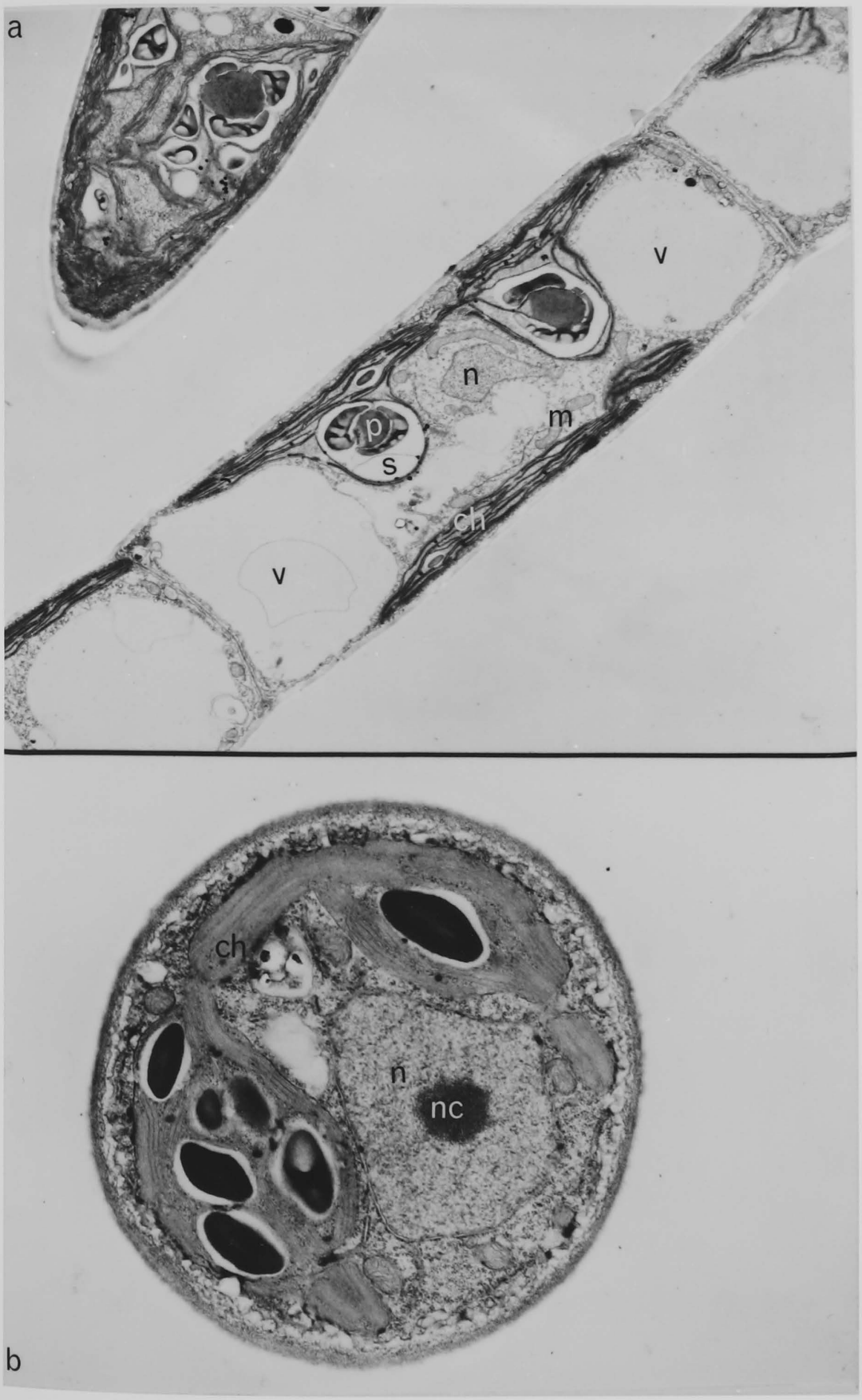
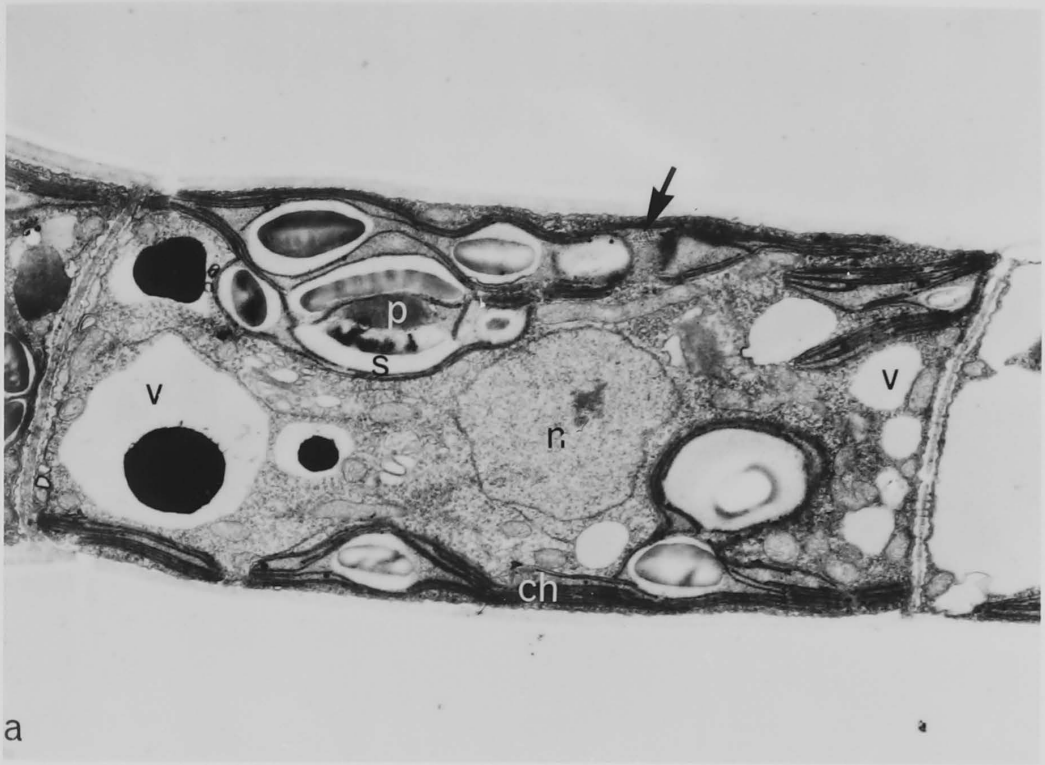

a
v
n
m
p
s
ch
v
ch
n
nc
b

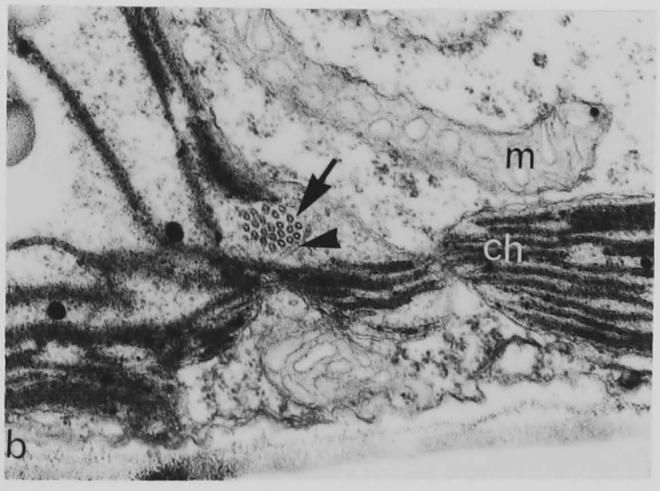
Fig. 2.7 Ultrastructure of Stigeoclonium cells.

(a) An older cell than that in the previous figure with pyrenoids (p) and starch (s) and a reduced vacuolar volume (v). Note the tubules (arrow) in the chloroplast (ch). (n, nucleus). X12,000.

(b) Tubules in the chloroplast (ch). The tubules are shown in both cross section (arrow) and longitudinal section (arrowhead). (m, mitochondrion). X34,000.




a



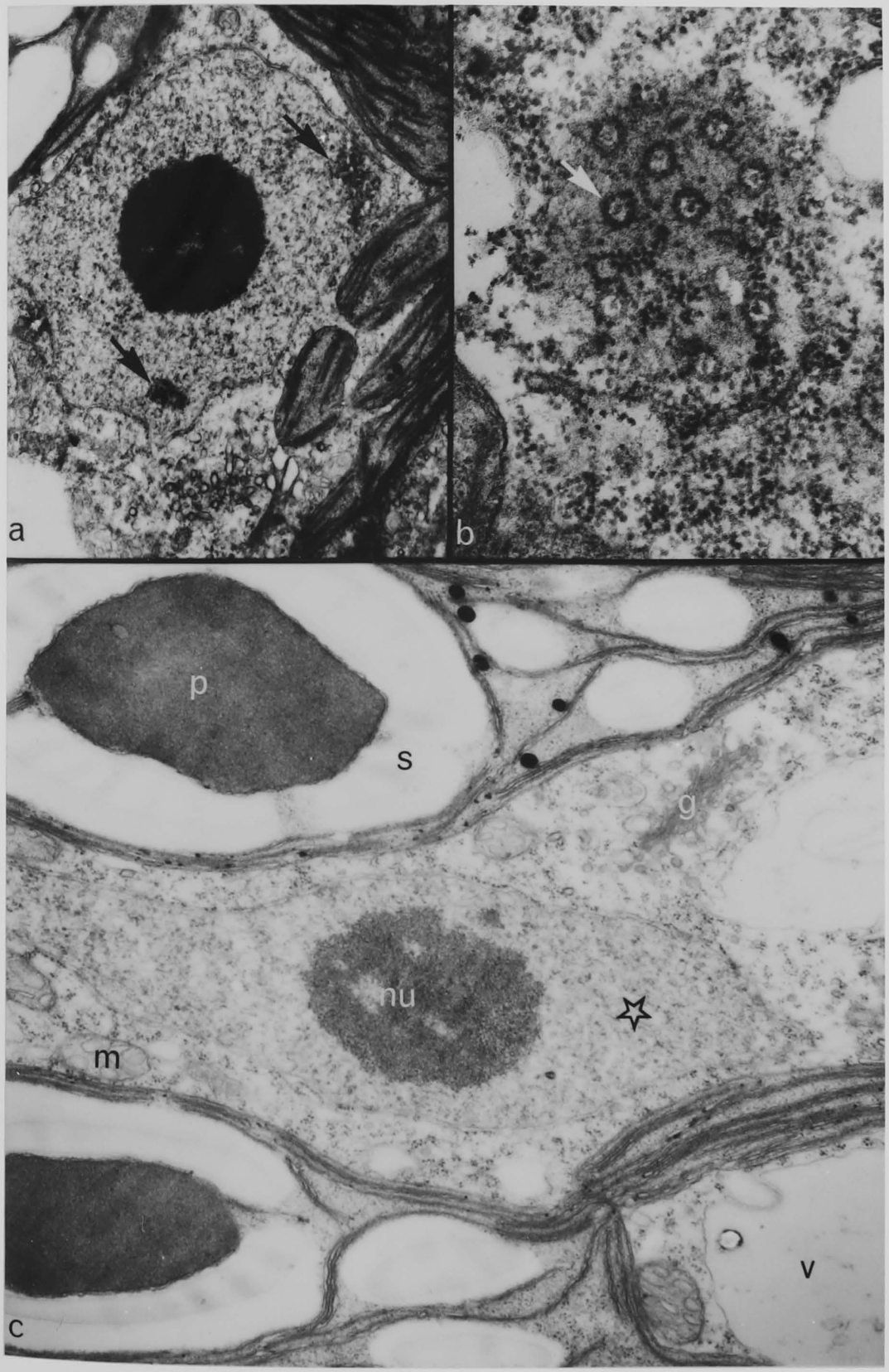

b

Fig. 2.8. Nuclear ultrastructure of Stigeoclonium.

(a) Discrete, densely staining bodies (arrowed) in a migrating nucleus. X16,250.

(b) Nuclear pores (arrow) in a section grazing the nuclear envelope of a migrating nucleus; note the central granule. X56,000.

(c) Migrating nucleus containing a very fine fibrillar matrix (starred). (p,pyrenoids; s, surrounding starch; v, vacuole; nu, nucleolus; m, mitochondria; g, golgi). X30,000.


a
b
p
s
g
nu
m
v
c

Conspicuous plasmodesmata traverse the septa. These plasmodesmata appear to be uniformly distributed over the septum. Although their diameter (30 nm) is similar to that of the plasmodesmata of higher plant cells they appear in most cases to lack a central tubule (desmotubule) (Fig. 2.9a). Occasionally plasmodesmata were observed with a possible desmotubule structure (Fig. 2.9b and c). The development of these algal cytoplasmic interconnections has not been investigated.

In older cells the septa often developed a band of opaque material and Y-shaped pieces at the ends with electron opaque deposits (Fig. 2.10a). The septa appear more resistant to enzymic digestion and remain as H-pieces after the digestion of the lateral cell walls during protoplast production (Fig. 2.10b) and zoospore release (Fig. 2.11d).

## 2.3.2 Life Cycle of Stigeoclonium

### (a) Asexual reproduction

Asexual reproduction by means of quadriflagellate zoospores is commonly observed particularly upon transfer of older filaments to fresh medium. The zoospores are often formed in cells situated between the apex and the first branch of the erect filament. Zoospores form from short cells with dense cytoplasm and are released after swelling and rupture of the parental cell wall (Fig. 2.11). Before their release, the zoospores were motile within their parental cell wall. Usually two,

Fig. 2.9 Plasmodesmata of Stigeoclonium.

(a) Simple, unbranched plasmodesmata (arrows) traversing the septum of two adjoining cells. (g, golgi; ch, chloroplast; m, mitochondria). X45,000.

(b) and (c)

Plasmodesmata showing a possible desmotubule structure (arrowhead) and with possible connections between endoplasmic reticulum (arrows) of adjacent cells. (b) X64,000; (c) X63,000.

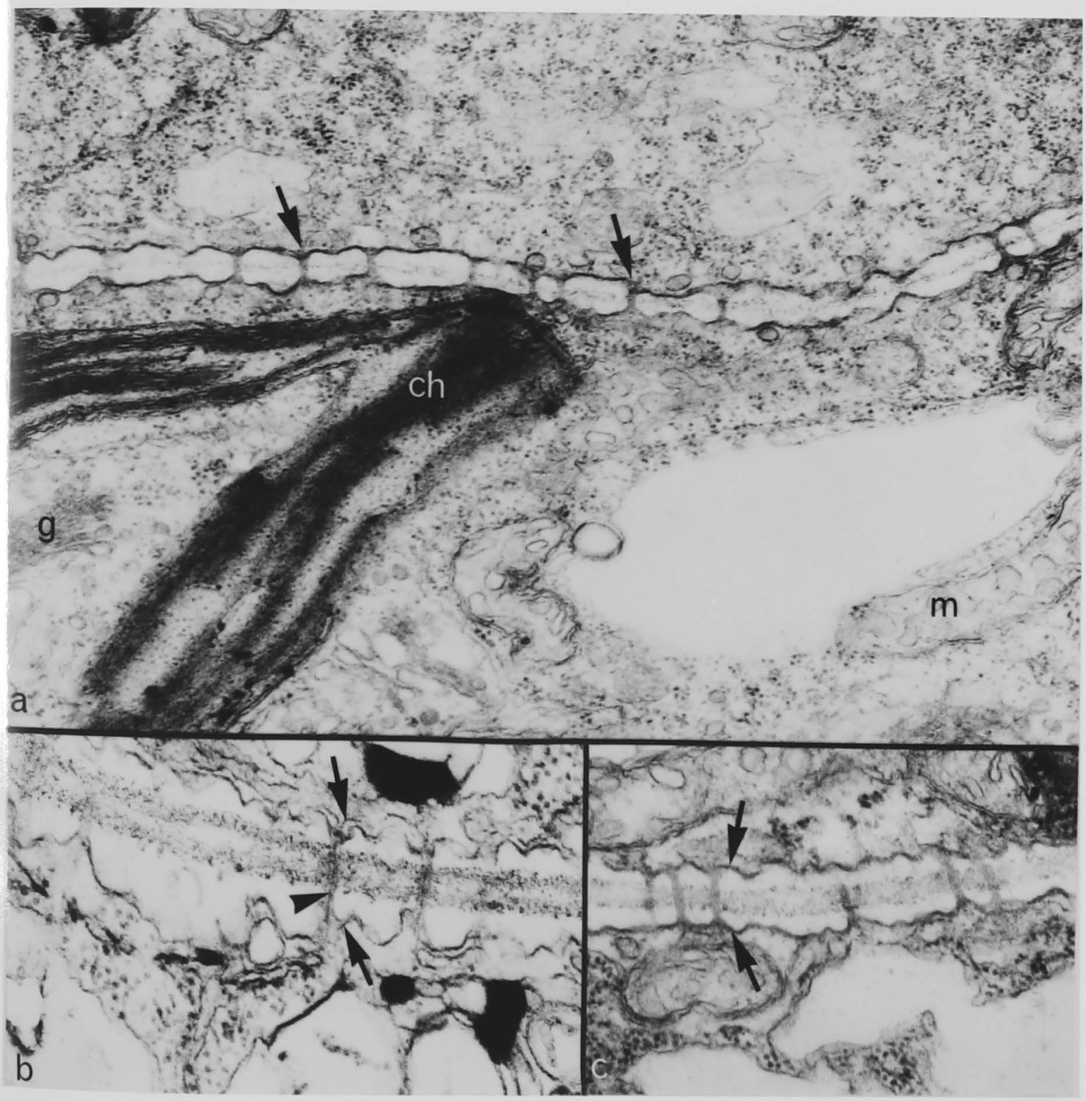

ch
g
m
a
b
c

Fig. 2.10 Septa of Stigeoclonium.

(a) An older cell wall showing Y-shaped pieces at the ends with electron opaque deposits (arrows). X19,000.

(b) H-pieces (arrowed) remaining after digestion of the lateral cell walls during protoplast production. Phase contrast X800.

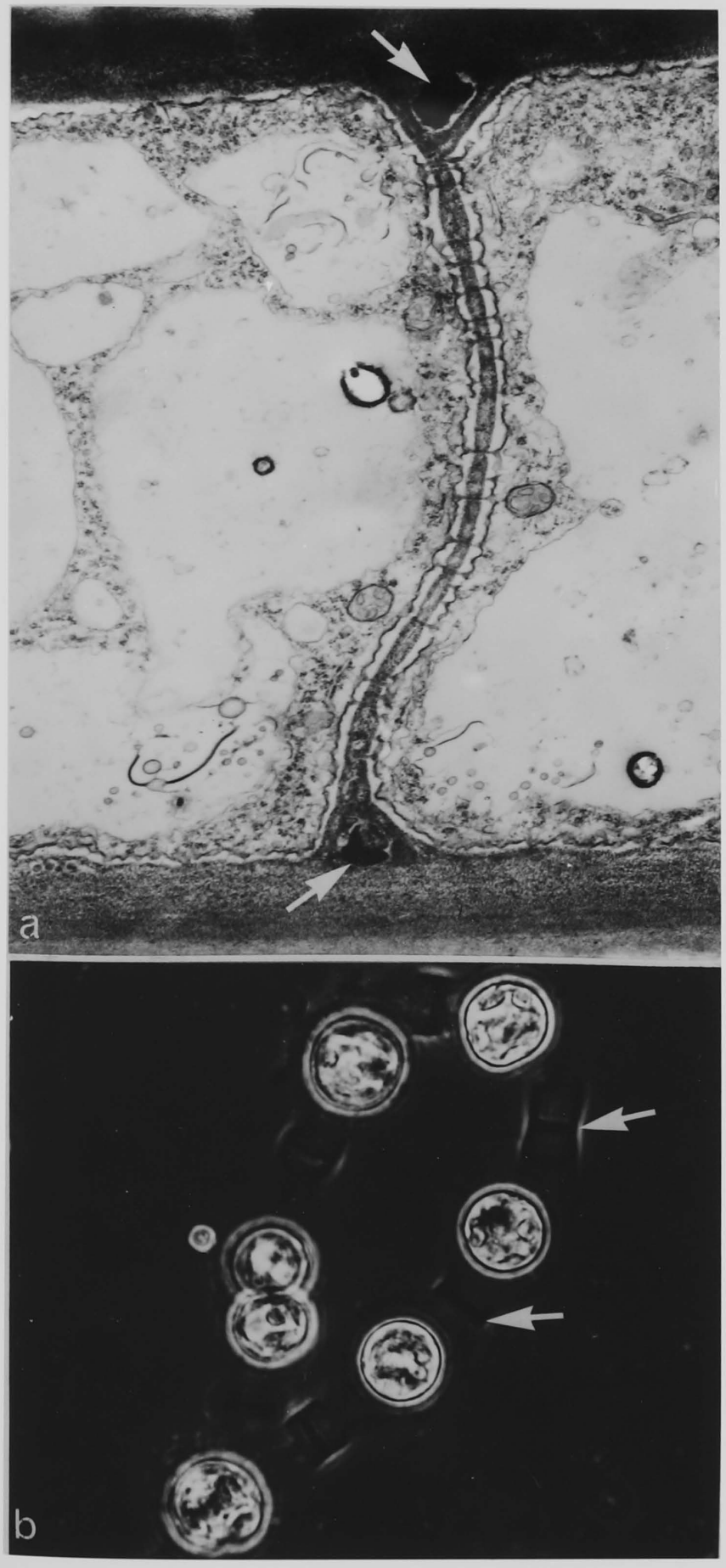

a
b

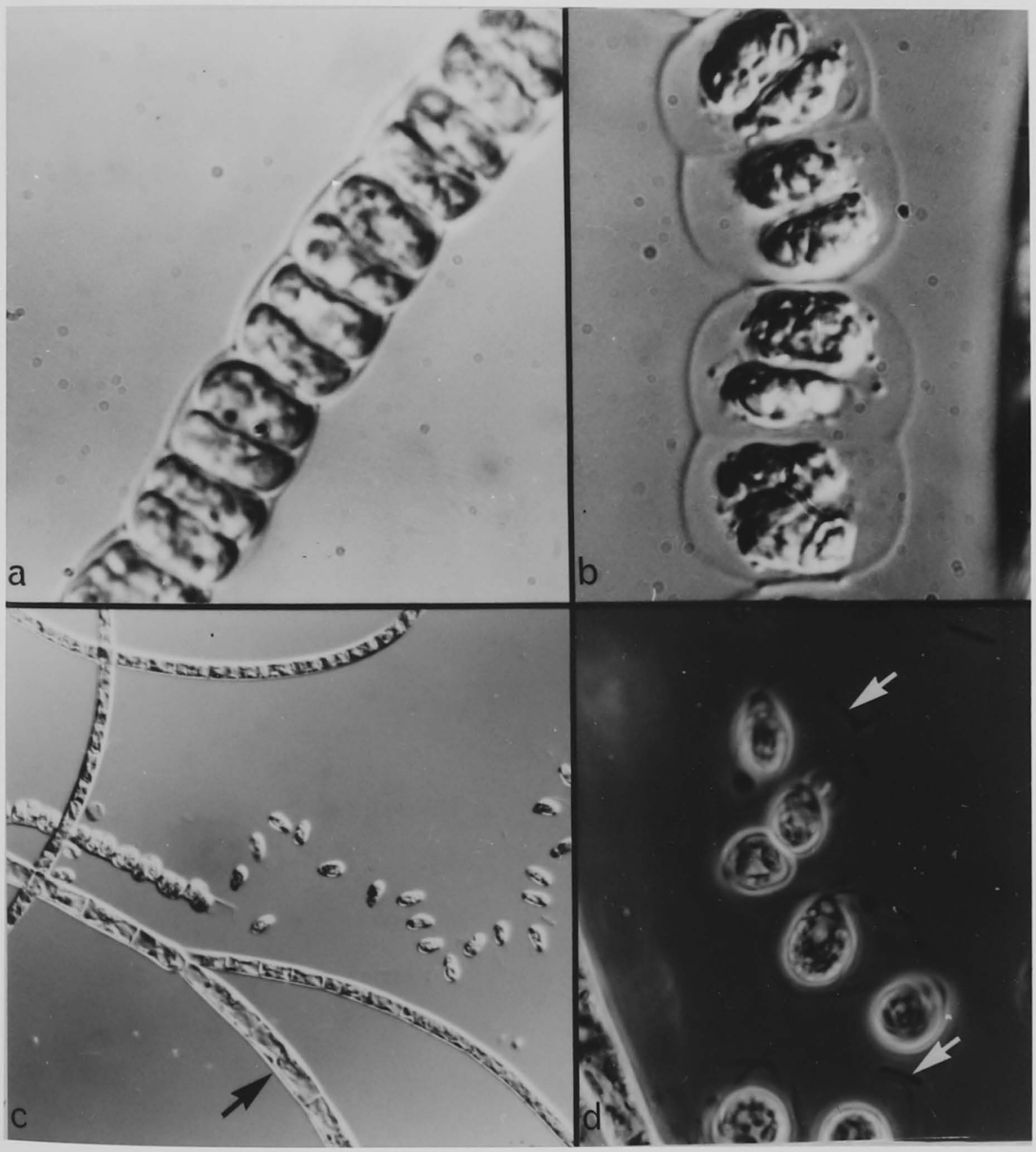
Fig. 2.11 Light micrographs of zoospore release.

(a) Barrel-shaped cells which are the first apparent stage of zoosporogenesis. Each cell contains two zoospores. Nomarski X1,400.

(b) Expansion of the cell wall just prior to release of the pair of zoospores. Nomarski X1,700.

(c) Release of zoospores following rupture of the parental cell wall. A normal vegetative cell is arrowed for comparison. Nomarski X350.

(d) Zoospores immediately after release from their parental cells. Release of zoospores follows degradation of the cell walls but the septa remain (arrow). Phase X850.


a
b
c
d

but sometimes one, zoospore was released from each cell. Cox and Bold (1966) observed a similar variation in the number of zoospores per cell. Cells of Stigeoclonium usually divide once every 24 hours at the beginning of the light period (see Section 2.3.4). However the first indication of zoosporogenesis is a decrease in the cell cycle time and the appearance of many short cells. Zoosporogenesis has not been studied in detail by electron microscopy; those few electron micrographs I have obtained confirm a radical cytoplasmic reorganisation in which the four flagella, having arisen symmetrically, occupy the corners of the parental cell (Fig. 2.12). The flagella contained the usual 9 + 2 doublet structure of the axoneme (Fig. 2.12b). During zoosporogenesis the plasmodesmata became attenuated and finally obliterated (Fig. 2.12c and d).

Manton (1964) has described the ultrastructure of Stigeoclonium zoospores fixed both when swimming and after settling. She suggested that after settling the persistent parts of the flagella bases may have a centrosomal function. Motile zoospores are very strongly phototactic. When movement stops the flagella are rapidly withdrawn and the cell secretes an adhesive material followed by a cell wall. The shape of settled zoospores changes dramatically, the contents become rearranged and eventually the nucleus divides.

(b) Sexual reproduction

Sexual reproduction has rarely been observed in Stigeoclonium and is poorly understood (Cox and Bold, 1966). Godward (1942) described sexual reproduction in Stigeoclonium amoenum Kutz. The vegetative cells

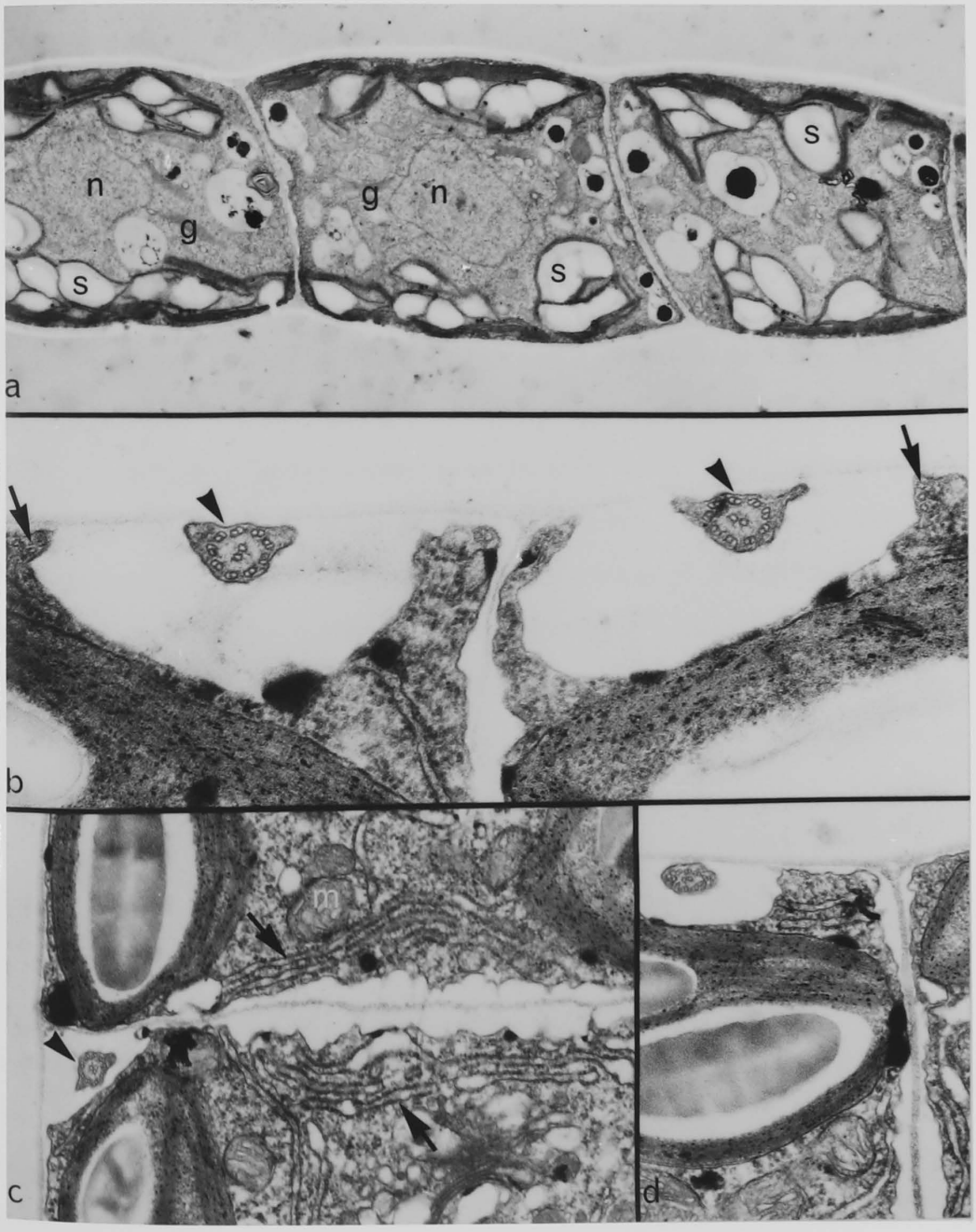
Fig. 2.12 Zoosporogenesis in Stigeoclonium.

(a) Short cells with dense cytoplasm, the first stage of zoosporogenesis. Note the oblique septa which are very rarely seen in vegetative cells. (n, nuclei; g, golgi; s, starch bodies). X8,500.

(b) Flagella (arrowed) of transversly sectioned zoospores with the usual 9 + 2 arrangement of axonemal microtubules at each 'corner' of the cell. Microtubules (arrowed) may represent part of the rootlet system. X60,000.

(c) Cell wall with tenuous plasmodesmata at a late stage of zoosporogenesis. (Flagellum, (arrowhead); hypertrophied rough endoplasmic reticulum (arrows); m, mitochondrion). X33,000.

(d) Late stage in zoosporogenesis with no apparent plasmodesmata. X35,000.


n
g
s
g
n
s
s
a
b
m
c
d

gave rise to gametes which fused to form a zygospore. During germination the contents of the zygospore divided forming four zoospores which were then released. During the course of my studies sexual reproduction was not observed.

## 2.3.3 Cell Size Analysis

The length and width of 1000 vegetative, inter-branch cells was measured to the nearest 2 µm with a calibrated eyepiece micrometer during the dark cycle (Fig. 2.13). Cell length showed considerable variation between 14 µm and 78 µm with the mode being 38 µm (Fig. 2.13a). Some of this variation in length may be due to differences in the timing of cell division and expansion in a single filament. By contrast cell width is strictly controlled, most cells being 8-10 µm wide (Fig. 2.13b). Thus the overall cross-wall area between cells may not vary greatly for young, vegetative cells of Stigeoclonium.

## 2.3.4 Cell Division in Stigeoclonium

The erect filaments of Stigeoclonium are observed to elongate by intercalary growth. The division of these cells adds a temporal component to the spatial control of pattern formation in Stigeoclonium. In this section the process of cytokinesis is described and the time of cytokinesis established.

### (a) Cytokinesis in Stigeoclonium

During the early stages of cytokinesis the vesicles of the new

Fig. 2.13 Cell Size Analysis

(a) The frequency distribution of cell length for 1000 cells.

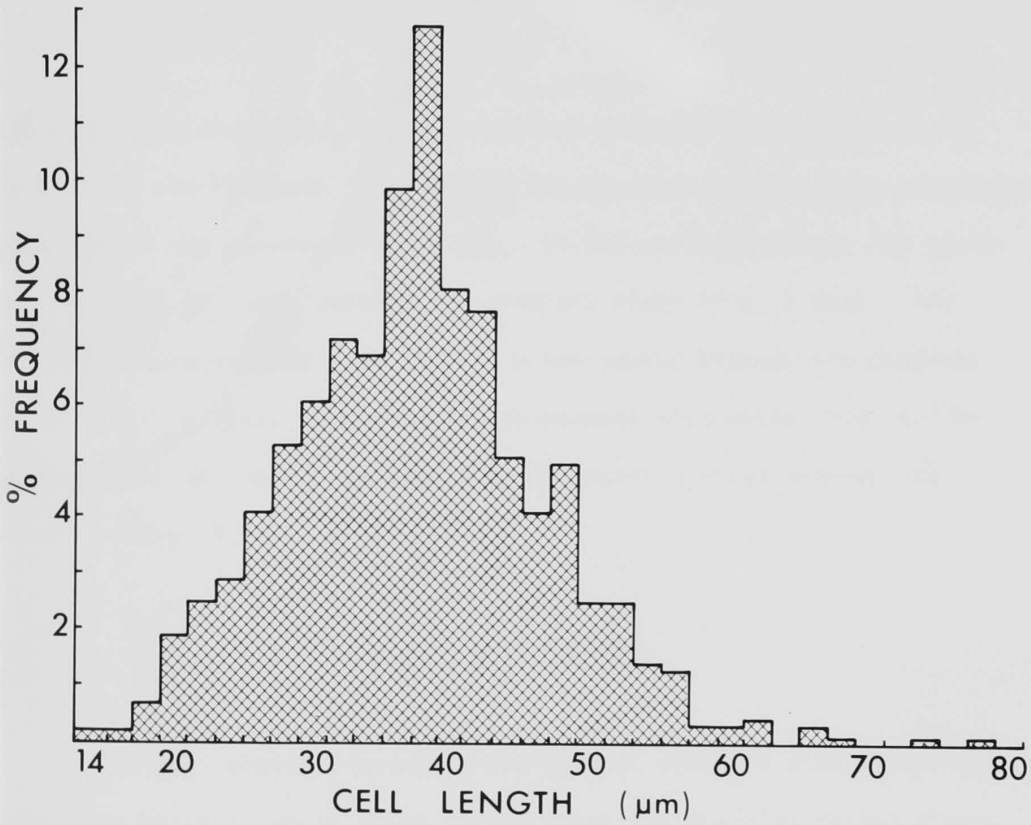
$\bar{x}$ = 34.84

s = 10.54

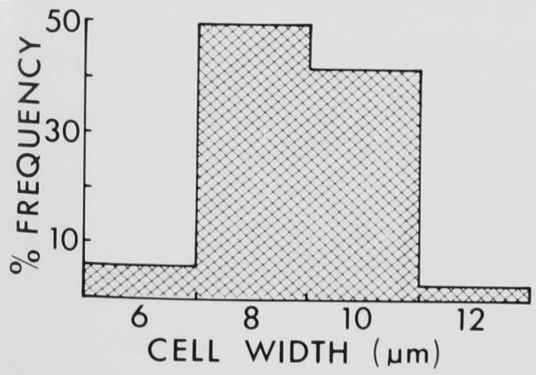
(b) The frequency distribution of cell width for 1000 cells.

$\bar{x}$ = 8.82

s = 1.29


% FREQUENCY
12
10
8
6
4
2
14
20
30
40
50
60
70
80
CELL LENGTH (µm)

a


% FREQUENCY
50
30
10
6
8
10
12
CELL WIDTH (µm)

b

(for definition see p. 93)
cell plate and phycoplast microtubules are aligned perpendicularly to the axis of the filament between the two daughter nuclei which are closely appressed to one another (Fig. 2.14). In the earliest stages the nuclei are flattened on their immediately adjacent sides (Fig. 2.14a). The vesicles accumulate and fuse to form a new septum between the daughter nuclei (Fig. 2.14b). The chloroplast becomes attenuated (Fig. 2.14b) in the region of the new cell plate and finally divides between the two cells (Fig. 2.15).

(b) Time of cell division

The cell plates present at the time of staining with Calcofluor fluoresced brightly while those formed after the staining period showed little fluorescence (Fig. 2.16b). The majority of cells had divided by 2100 hours, four hours into the light period (Fig. 2.16a). Cell division is entrained to the light-dark cycle with an increase in the frequency of cytokinesis beginning just before the start of the light period. The initiation of branching apparently occurs soon after cytokinesis and this is discussed fully in the next section.

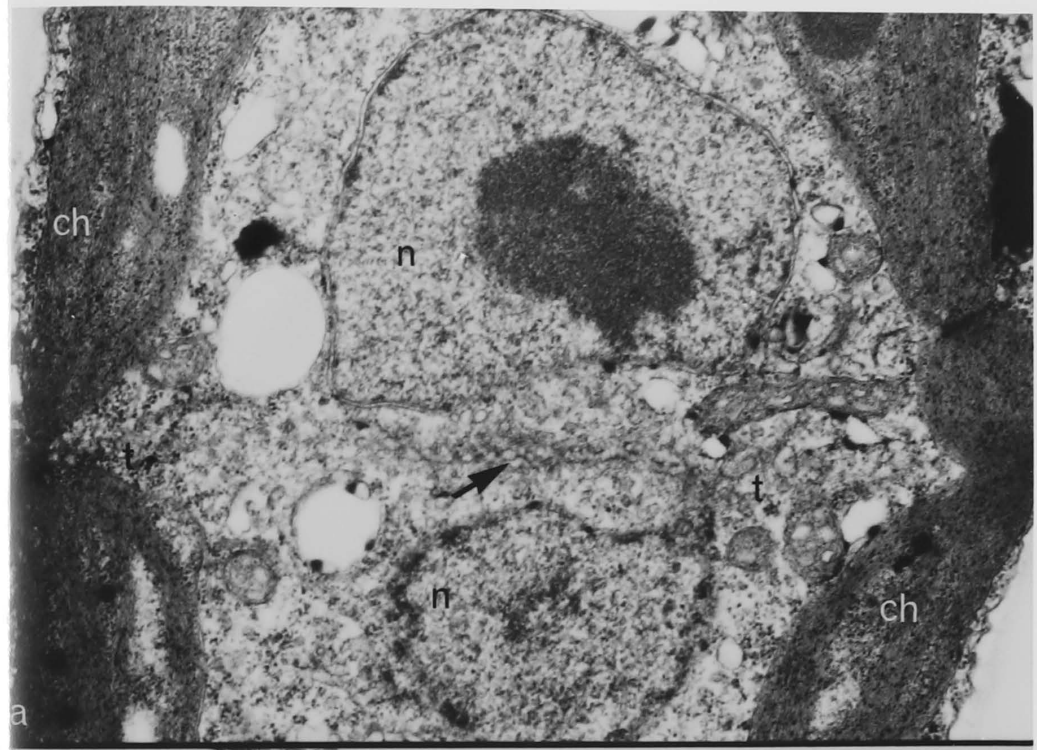
## 2.3.5 Branch Development in Stigeoclonium

The branching pattern in Stigeoclonium results from a dynamic equilibrium between branch initiation and cell division. New branches are initiated between existing branches and subsequently spaced out by cell division (Fig. 2.17). The dynamic nature of pattern formation in Stigeoclonium makes it important to study the ontogenetic aspects of

Fig. 2.14 Cytokinesis in Stigeoclonium

(a) An early stage in the alignment of vesicles (arrow) and microtubules (t). Note the proximity of the nuclei and their flattening on one side. (n, nuclei; ch, chloroplasts). X30,000.

(b) Accumulation and fusion of vesicles to form a new septum. The chloroplast (ch) is attenuated in the region of the new cell plate (arrowed). (g, golgi; n, nuclei). X27,000.


ch
n
t
t
n
ch
a

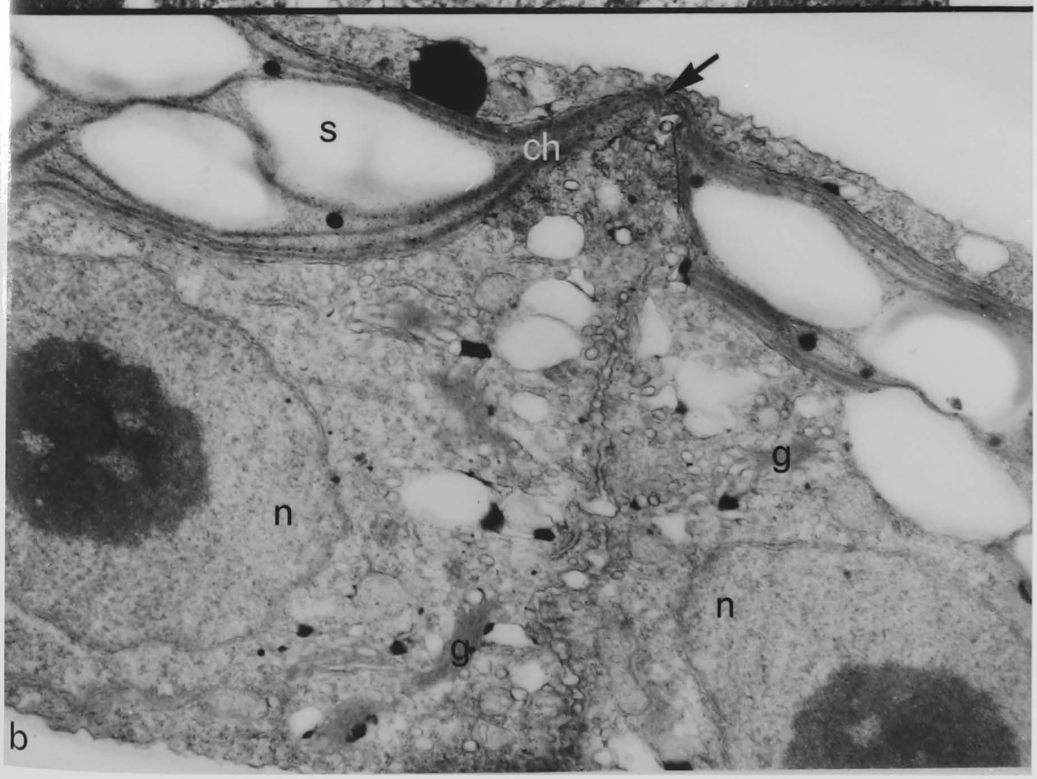

s
ch
g
n
n
g
b

Fig. 2.15 Cytokinesis in Stigeoclonium (cont.)

(a) Extensive fusion of vesicles has occured but some gaps (arrow) remain in the cell plate. Note the tubules in longitudinal section (arrowhead). (n, nucleus; g, golgi) X22,000.

(b) A later stage of cell plate (arrow) formation where cytokinesis is almost complete. (n, nucleus) X24,000.

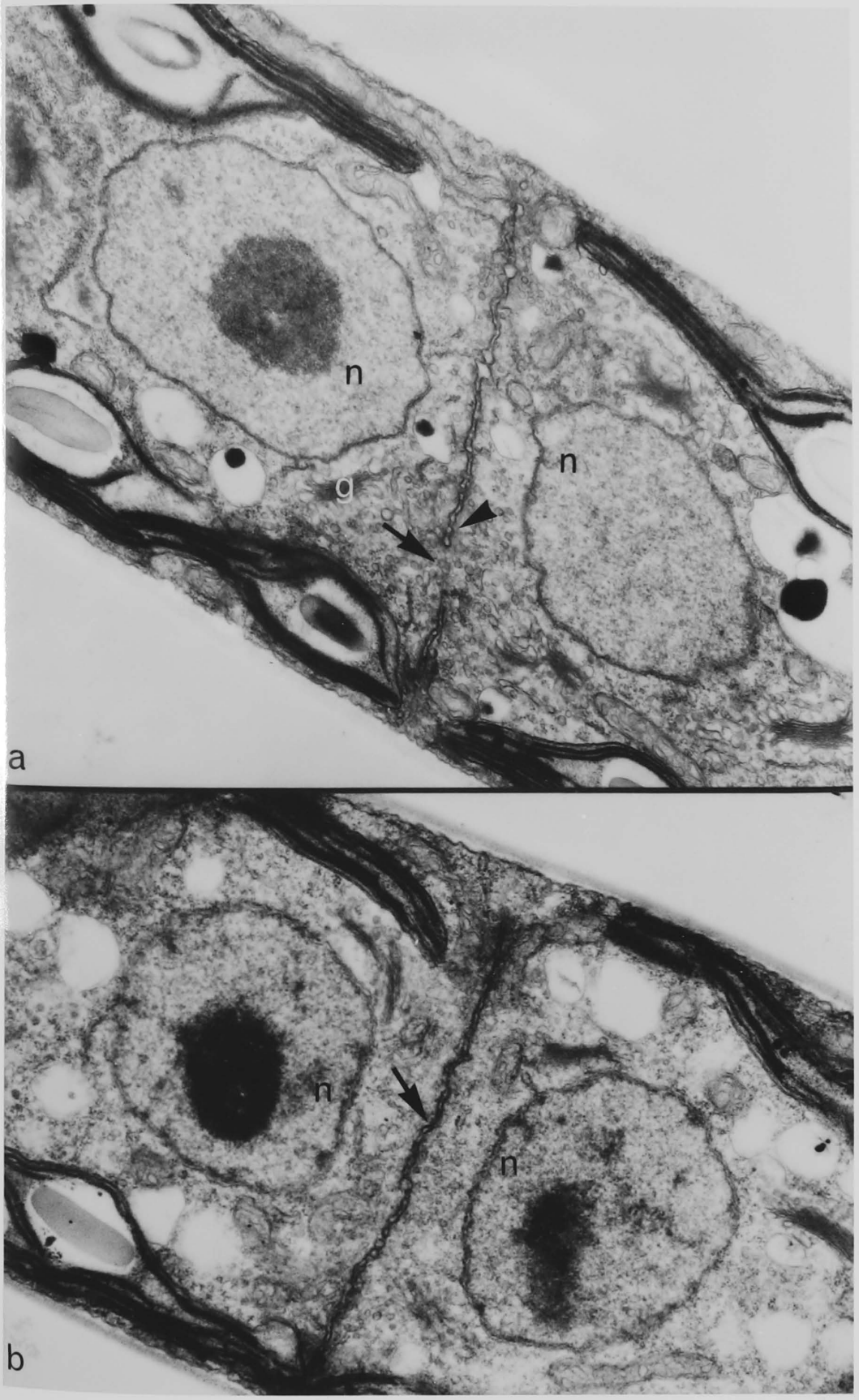

n
n
g
a
n
n
b

Fig. 2.16 The timing of cytokinesis in Stigeoclonium.

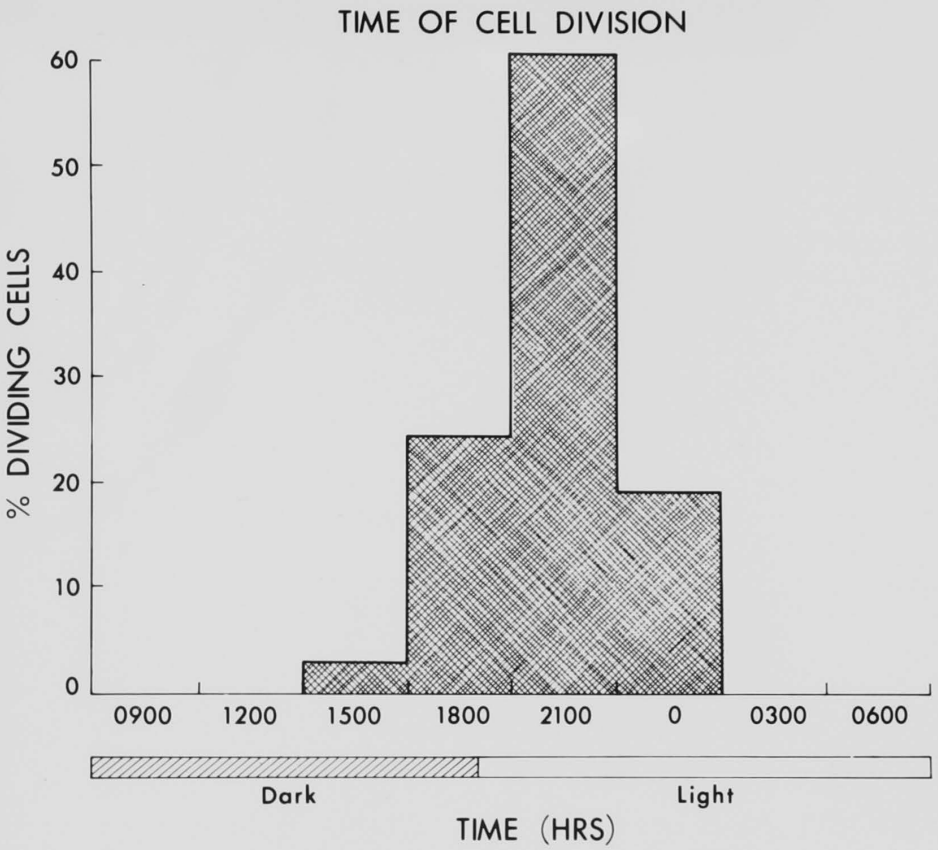
(a) The number of cells that have divided (200 cells examined at each time) within the previous 3 hours over a 24 hour period. Cytokinesis is entrained to the light/dark cycle with most cells having divided by 2100 hours, four hours after the onset of light.

Dark : 0900 to 1700 hrs.

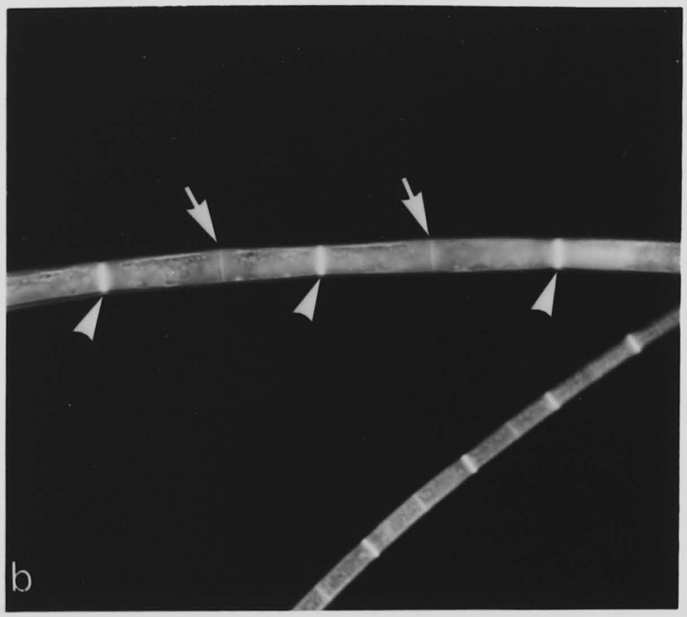
Light :1700 to 0900 hrs.

The horizontal axis represents the time of recording the number of cells that had divided within the previous three hours.

(b) Use of Calcofluor staining to demonstrate the sequence of cytokinesis. The new septa (arrows) appear faintly between the bright fluorescence of the old septa (arrowheads). This demonstrates intercalary growth of the filament. Combined phase contrast and ultraviolet fluorescence microscopy X350.


TIME OF CELL DIVISION
% DIVIDING CELLS
60
50
40
30
20
10
0
0900
1200
1500
1800
2100
0
0300
0600
Dark
Light
TIME (HRS)

a



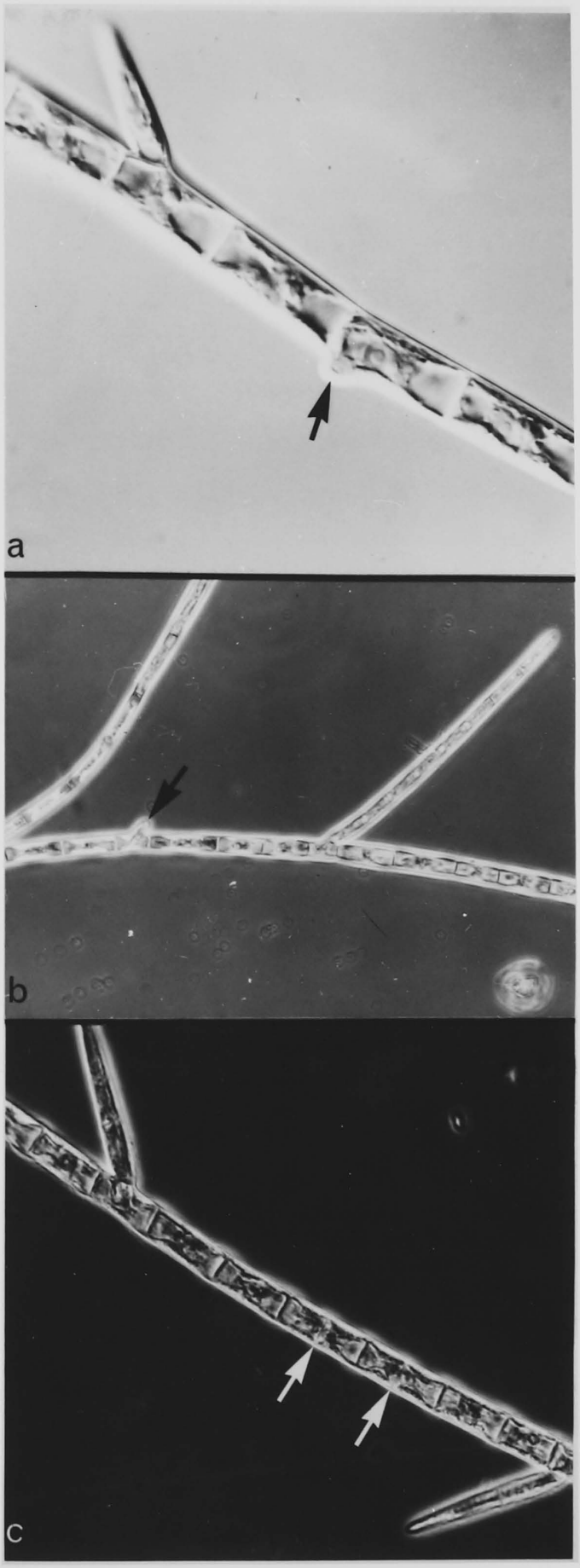
b

Fig. 2.17 The branch pattern as a dynamic equilibrium between branch initiation and cell division.

(a) Initiation of a new branch (arrow) only one cell away from an existing branch. Nomarski X700.

(b) Initiation of a new branch (arrow) between two established branches. Phase contrast X250.

(c) Two recent cell divisions (arrows) have increased the spacing between the branches from 6-cells to 8-cells. Phase contrast X500.


a
b
c

branching and this is detailed in the next section.

(a) Ontogenetic studies of branching in Stigeoclonium

Initially, there are no branches along the erect filament. Branch development usually starts after 7-10 days of culture. The position from which the first branch arises along any one erect filament, appears to be random (Table 2.2). This was determined by counting back from the apex of young filaments which had only one branch. This first branch usually arose from a cell which had divided before the adjacent cells in the filament (Table 2.3). As cultures become older the branches become more common but they generally do not become closer together than a critical number of cells (see section 2.3.6). It appears that there has to be a certain number of branches before a definite pattern in their spacing emerges.

The effect of branch age on pattern formation in Stigeoclonium

The division of the cells in the erect filament was studied by using Calcofluor during the development of the branch. The existing cell plates and branches fluoresced brightly after staining with Calcofluor while any new cell plate and branch formed after the staining period showed little fluorescence. Thus the technique allowed *in vivo* monitoring of cell division and branch formation. The data (Table 2.4) clearly show that after the branch has developed beyond the two cell stage the basal cell bearing it in the erect filament ceases to divide transversely to the axis of the filament. Cells adjacent to the basal cell continued to divide in the main axis of the filament.

TABLE 2.2 THE POSITION OF THE FIRST BRANCH IN YOUNG FILAMENTS WITH A SINGLE BRANCH

| No.of Cells from Apex To First Branch | % Frequency (100 Counts) |
|---|---|
| 1 | 0 |
| 2 | 0 |
| 3 | 4 |
| 4 | 5 |
| 5 | 2 |
| 6 | 3 |
| 7 | 2 |
| 8 | 2 |
| 9 | 0 |
| 10 | 4 |
| 11 | 2 |
| 12 | 3 |
| 13 | 5 |
| 14 | 1 |
| 15 | 5 |
| 16 | 4 |
| 17 | 3 |
| 18 | 3 |
| 19 | 5 |
| 20 | 3 |
| 21 | 2 |
| 22 | 5 |
| 23 | 3 |
| 24 | 3 |
| 25 | 4 |
| 26 | 3 |
| 27 | 6 |
| 28 | 5 |
| 29 | 1 |
| 30 | 2 |
| 31 | 6 |
| 32 | 4 |

TABLE 2.3 SYNCHRONY OF CELL DIVISION AND THE INITIATION OF THE FIRST BRANCH IN A YOUNG FILAMENT

| Cell division of the branch initial in relation to adjacent cells | |
|---|---|
| (100 branches counted) | |
| Synchronous | Asynchronous |
| 21 | 79 |

TABLE 2.4 THE EFFECT OF BRANCH AGE ON THE DIVISION OF THE BASAL CELL.

| No. of Cells In Branch | Division of Basal Cell in the main axis. % frequency (100 Counts) |
|---|---|
| 0 | 81 |
| 1 | 53 |
| 2 | 26 |
| 3 | 0 |
| 4 | 0 |
| 5 | 0 |
| 6 | 0 |
| 7 | 0 |
| 8 | 0 |
| 9 | 0 |
| 10 | 0 |

The timing of branch initiation in Stigeoclonium.

By the use of Calcofluor the time of branch initiation (Table 2.5) could be correlated with the time of cytokinesis (Fig. 2.16). Just as with cytokinesis branch initiation occurs over some hours, but is most frequent around the time of maximum cytokinesis. From this it could be concluded that the majority of branches are formed soon after cytokinesis is completed.

(b) Morphology of Branch Development in Stigeoclonium

During branch development a large vacuole was formed at the opposite end of the cell (Figs. 2.18b and c). As the branch elongates the nucleus comes to lie at the branch point where it will divide (Fig. 2.18d). The initiating cell of a mature branch becomes differentiated from the normal axial cells of the filament by developing a thick wall and a barrel shape (Fig. 2.18e). The apical cell of a mature branch develops hyaline hair cells (Fig. 2.18f). Hair cells may also arise from young branches in old cultures presumably depleted of nutrients (Fig. 2.18g). In old cultures double branch points were also formed by initiation of a second branch from a basal cell which already bore a mature branch (Figs. 2.19). Very rarely branches were formed from cells immediately adjacent to a double branch point (Fig. 2.19d).

Ultrastructurally branch initiation consisted of a protrusion at one end of the cell coupled with a displacement of the nucleus and associated cytoplasm towards that end and the formation of a large vacuole at the opposite end (Fig. 2.20a). High magnification revealed

TABLE 2.5 THE TIMING OF BRANCH INITIATION IN STIGEOCLONIUM.

| Time (Hours) * | % New Branches Formed (100 cells counted at each time) |
|---|---|
| 0900 (D) | 5 |
| 1200 (D) | 3 |
| 1500 (D) | 8 |
| 1800 (L) | 10 |
| 2100 (L) | 23 |
| 0000 (L) | 6 |
| 0300 (L) | 2 |
| 0600 (L) | 3 |

* Light (L)/Dark (D) Cycle:

Light from 1700 to 0900 hours.
Dark from 0900 to 1700 hours.

Fig. 2.18 Branch Development in Stigeoclonium.

(a) Normal vegetative cell with central nucleus (arrow) and two vacuoles (v), one at either end of the cell. Nomarski X950.

(b) and (c)
Branch development showing the single large vacuole (v) and movement of the cytoplasm into the branch.
(b) Nomarski X850. (c) Nomarski X850.

(d) A developing branch with the nucleus (arrow) positioned in readiness for mitosis and cytokinesis. Nomarski X1,200.

(e) The thick walled, barrel-shaped basal cell (arrow) of a mature branch. Nomarski X500.

(f) Hair cells (arrows) formed at the apex of mature branches. Phase Contrast X500.

(g) Hair cell formed by a young branch in an old culture.
Phase Contrast X250.

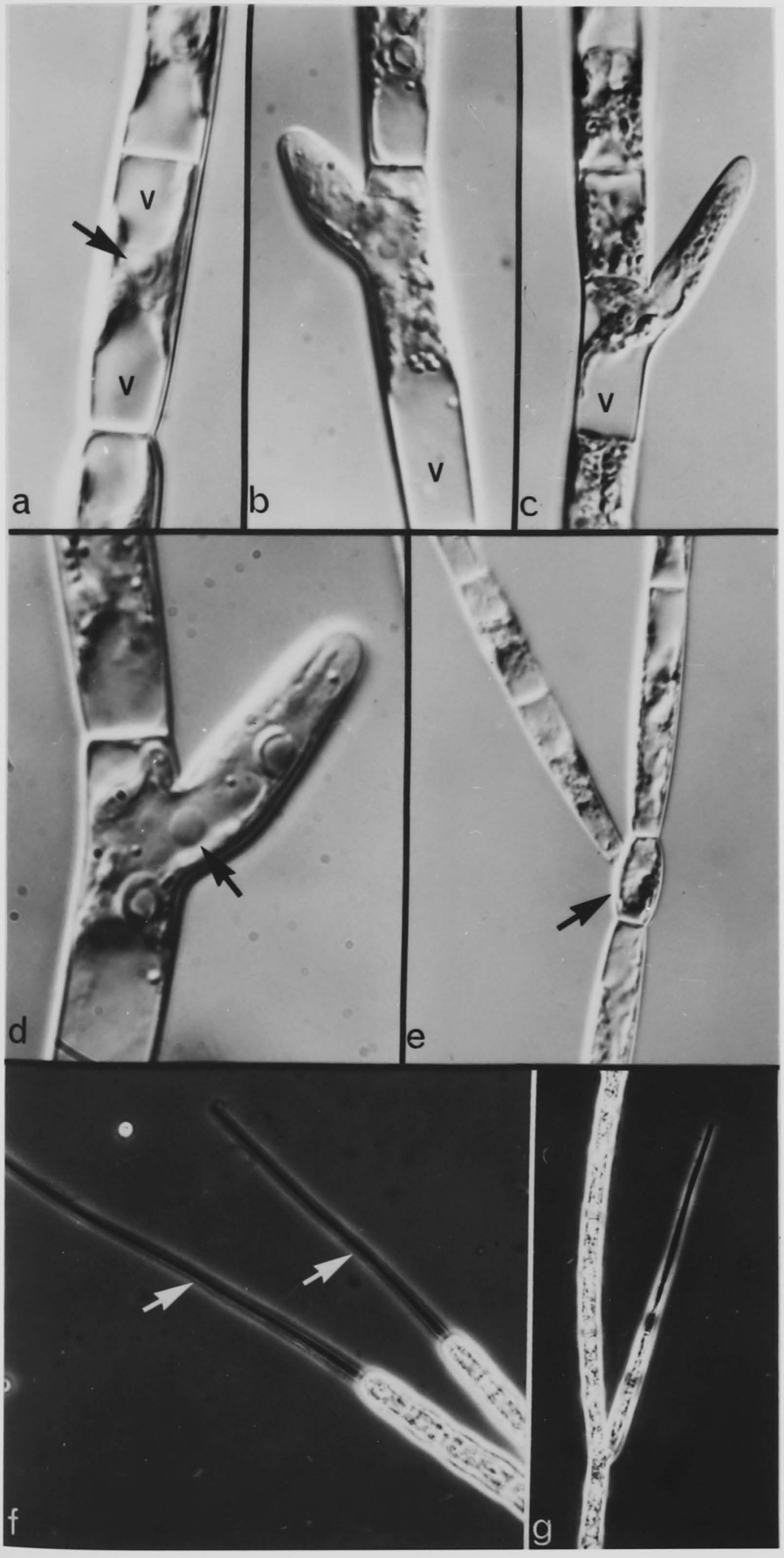

a
v
v
b
v
c
v
d
e
f
g

Fig. 2.19 Development of a double branch point.

(a) and (b) Initiation and growth of the second branch (arrows). Both Phase contrast X600.

(c) A mature double branch point. Phase contrast X250.

(d) Development of a branch (arrowed) from a cell adjacent to a double branch point. Phase contrast X500.

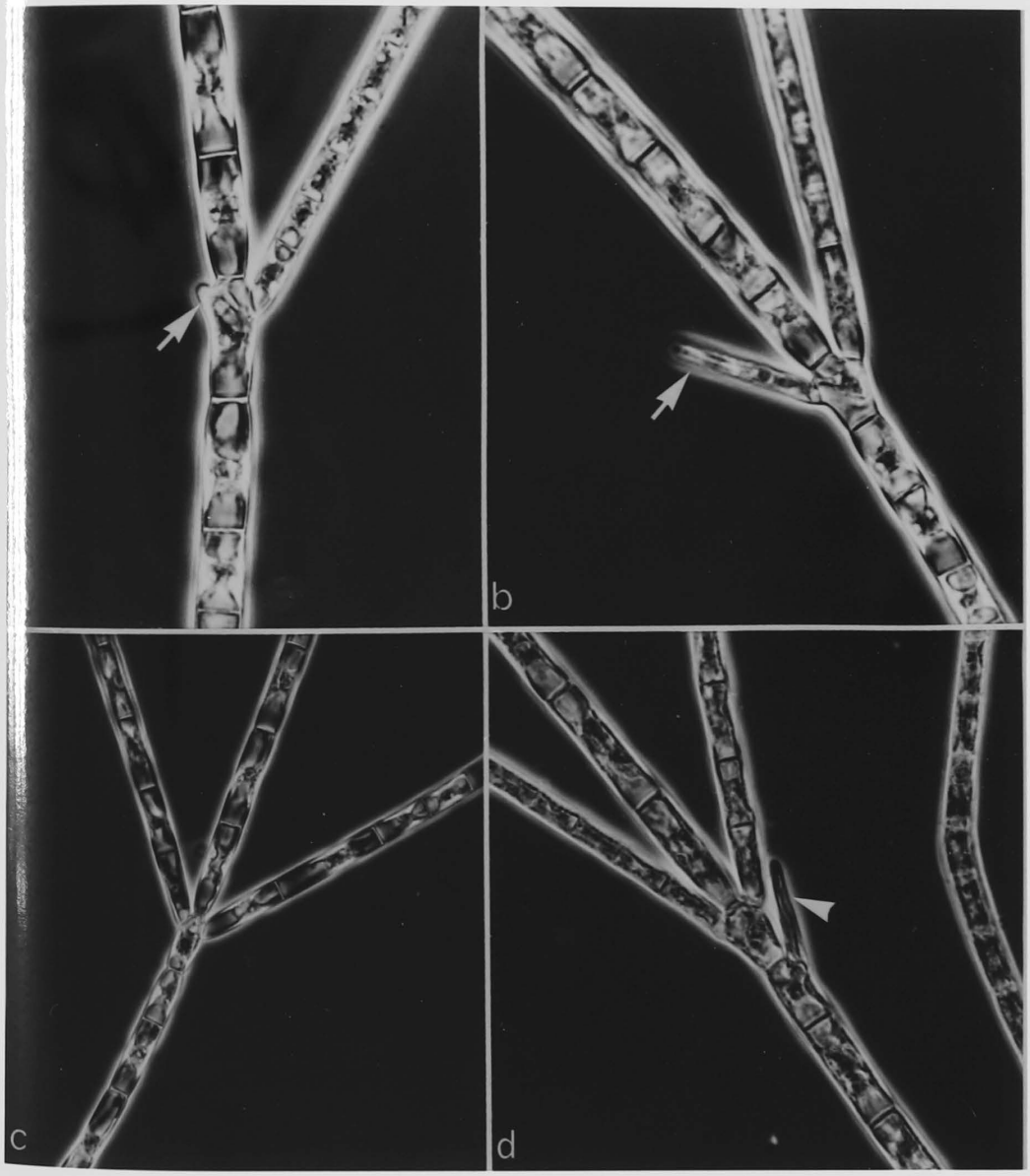
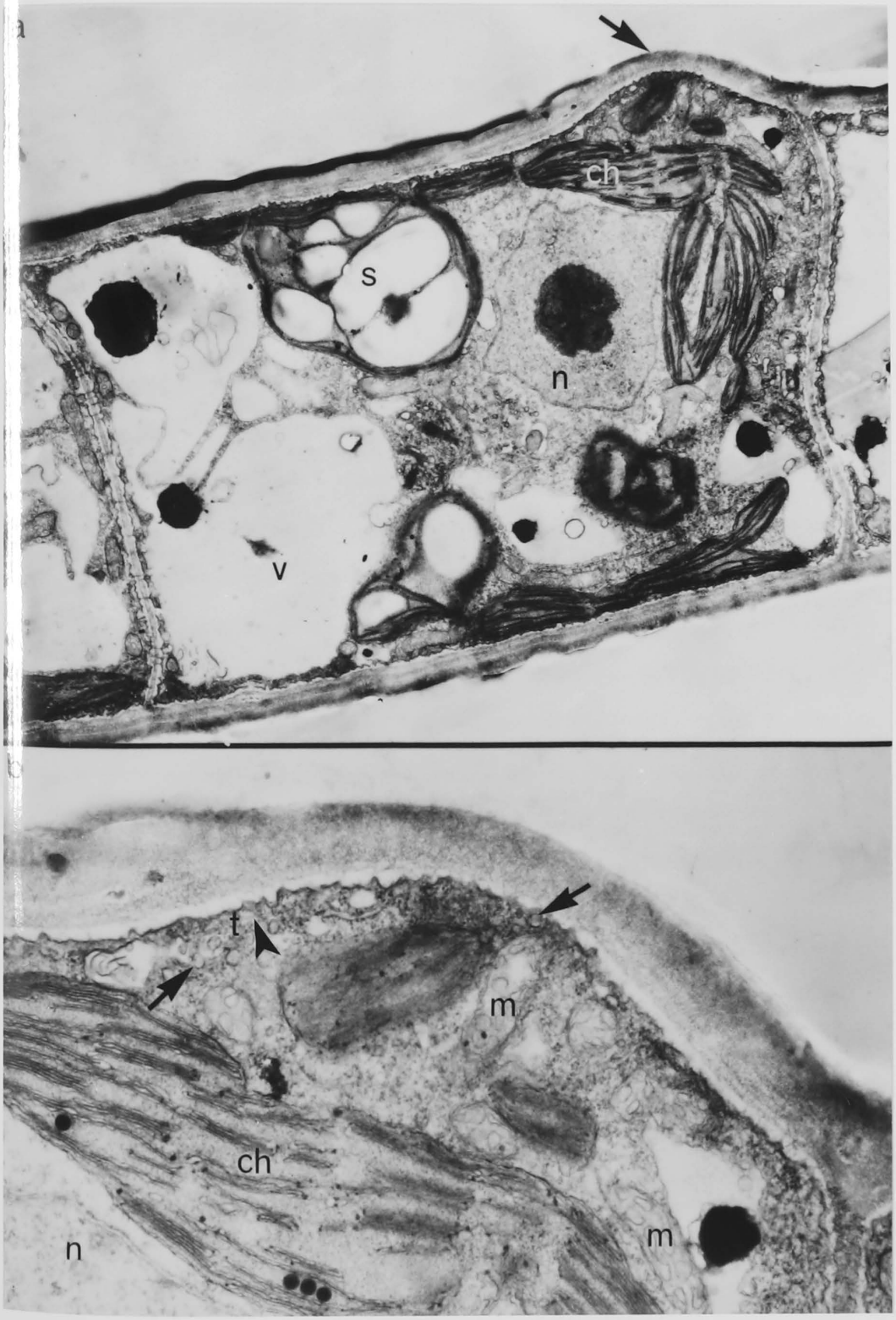

b
c
d

Fig. 2.20 Ultrastructure of branch development in Stigeoclonium.

(a) First stages of branch initiation show a displacement of the nucleus towards one end of the cell and the formation of a large vacuole (v) at the opposite end of the cell. Note the protrusion at the end of the cell (arrow). (ch,chloroplast; s,starch bodies). X9,500.

(b) High magnification of an early stage of branch initiation. Note the bulging of the cell wall and the concentration of vesicles (arrows). (ch, chloroplast; n, nucleus; m, mitochondria; t,microtubule, arrowhead). X31,000.


ch
s
n
v
t
m
ch
n
m

a concentration of vesicles under the bulge of the cell wall (Fig.2.20b), the branch initial. These vesicles would be involved in both softening of the existing cell wall and deposition of new cell wall materials to be incorporated into the developing branch. As the branch elongates further, organelles such as the mitochondria, golgi apparatus and the chloroplast migrate into the branch (Fig. 2.21). As the branch further develops the nucleus positions itself at the branch point where it will undergo mitosis and the septum will be deposited (Fig. 2.22). This premitotic nucleus was associated with centrioles at opposite poles near the nuclear envelope (Fig. 2.23). After formation of the cell plate the daughter nuclei retreat from their positions adjacent to the cell plate (Fig. 2.24). The apex of the elongating branch shows marked vesiculation (Fig. 2.25a) and the presence of microtubules (Figs. 2.25b and c). The apex of a developing branch also has extensive vesicles, golgi bodies, abundant cytoplasmic ribosomes and endoplasmic reticulum (Fig. 2.26).

Fig. 2.21 Branch Development in Stigeoclonium.

(a) and (b) Movement of cellular organelles such as mitochondria (m), golgi apparatus (g), and the chloroplast (ch) into the developing branch. Compare the reduced vacuole (v) in the branching cell with the large vacuole (V) in the adjacent cell in (a).

(p, pyrenoids; n, nucleus; v, vacuoles; s, starch bodies).

(a) X14,000 and (b) X13,000.


ch
m
n
p
v
V
a


ch
m
g
n
s
v
v
b

Fig. 2.22 Branch Development in Stigeoclonium.

(a) and (b) Extension of the branch and movement of the nucleus into the position where it will undergo mitosis and the new cell plate will be formed.

(n, nucleus; nc, nucleolus;
v, vacuole; ch, chloroplast;
p, pyrenoid; s, starch;
m, mitochondria; g, golgi).

(a) X12,500 and (b) X11,000.

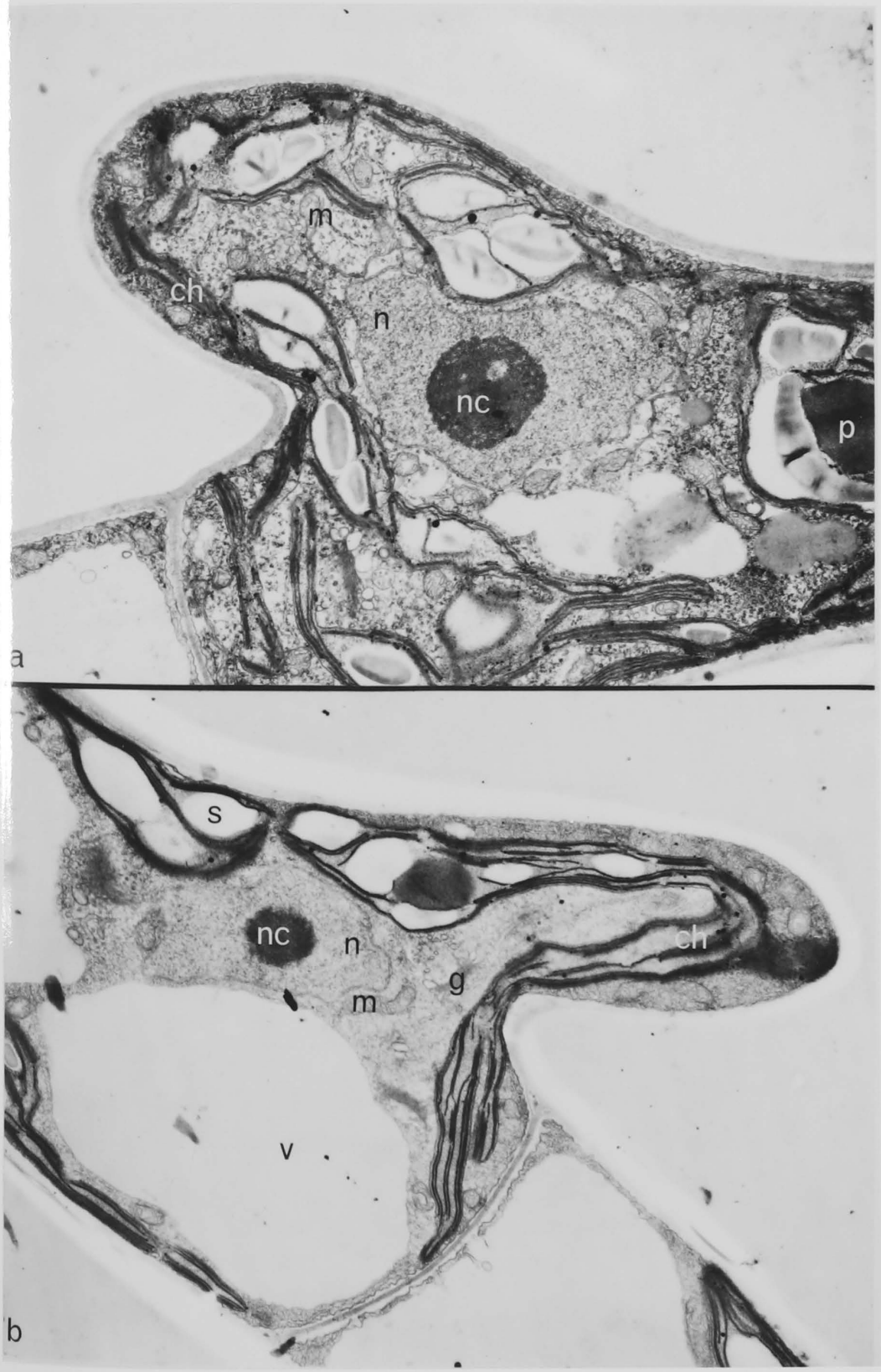

m
ch
n
nc
p
a
s
nc
n
ch
g
m
v
b

Fig. 2.23 Branch Development in Stigeoclonium.

(a) Placement of the nucleus (n) prior to its division and cytokinesis. There are two and possibly three centrioles (arrow) adjacent to the nuclear envelope. Note the large vacuole (v) at the basal end of the cell. X7,200.

(b) Premitotic nucleus (n) with a pair of centrioles (arrows) at one pole, and a single centriole at the opposite pole (arrow). This nucleus lies at the base of a developing branch. (nc, nucleolus). X20,000.

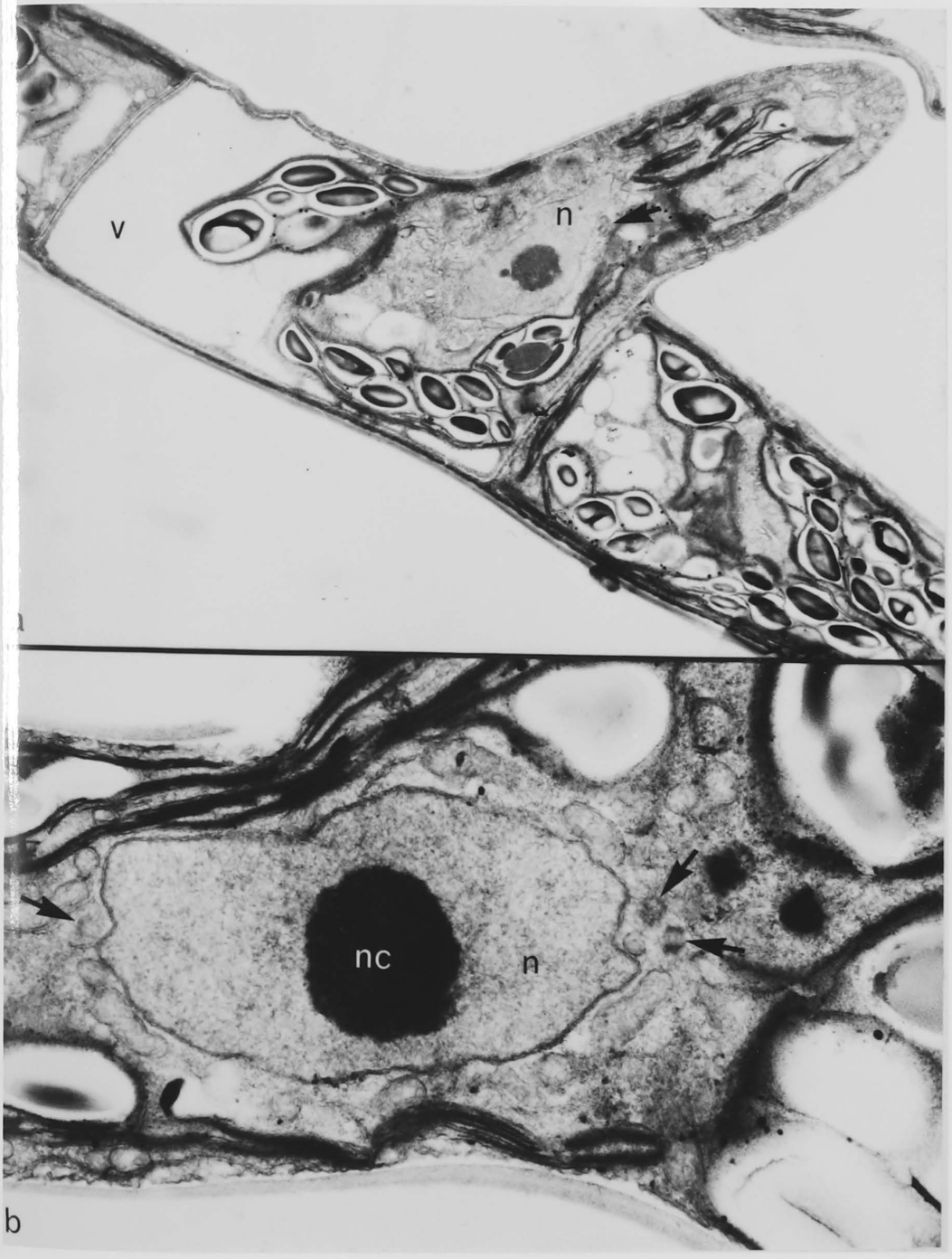

v
n
nc
n
b

Fig. 2.24 Branch Development in Stigeoclonium.

Formation of the cell plate between the new branch and the cell bearing it. The daughter nuclei (n) have retreated from their position adjacent to the cell plate. The new cell plate (arrow) lacks the dark staining of the old cell wall (arrowhead).

X17,500.


n
ch
m
g
n
v

Fig. 2.25 Apical region of a developing branch in *Stigeoclonium*.

(a) Incorporation of new wall material. Note the vesiculation (arrowed) and the centriole complex (c). (n, nucleus; g, golgi; ch, chloroplast; m, mitochondria).

(b) and (c) Microtubules in longitudinal and cross section (arrowed) near the apex of a developing branch. (m, mitochondria; ch, chloroplast; c, cell wall). (b) X57,000 and (c) X36,000.

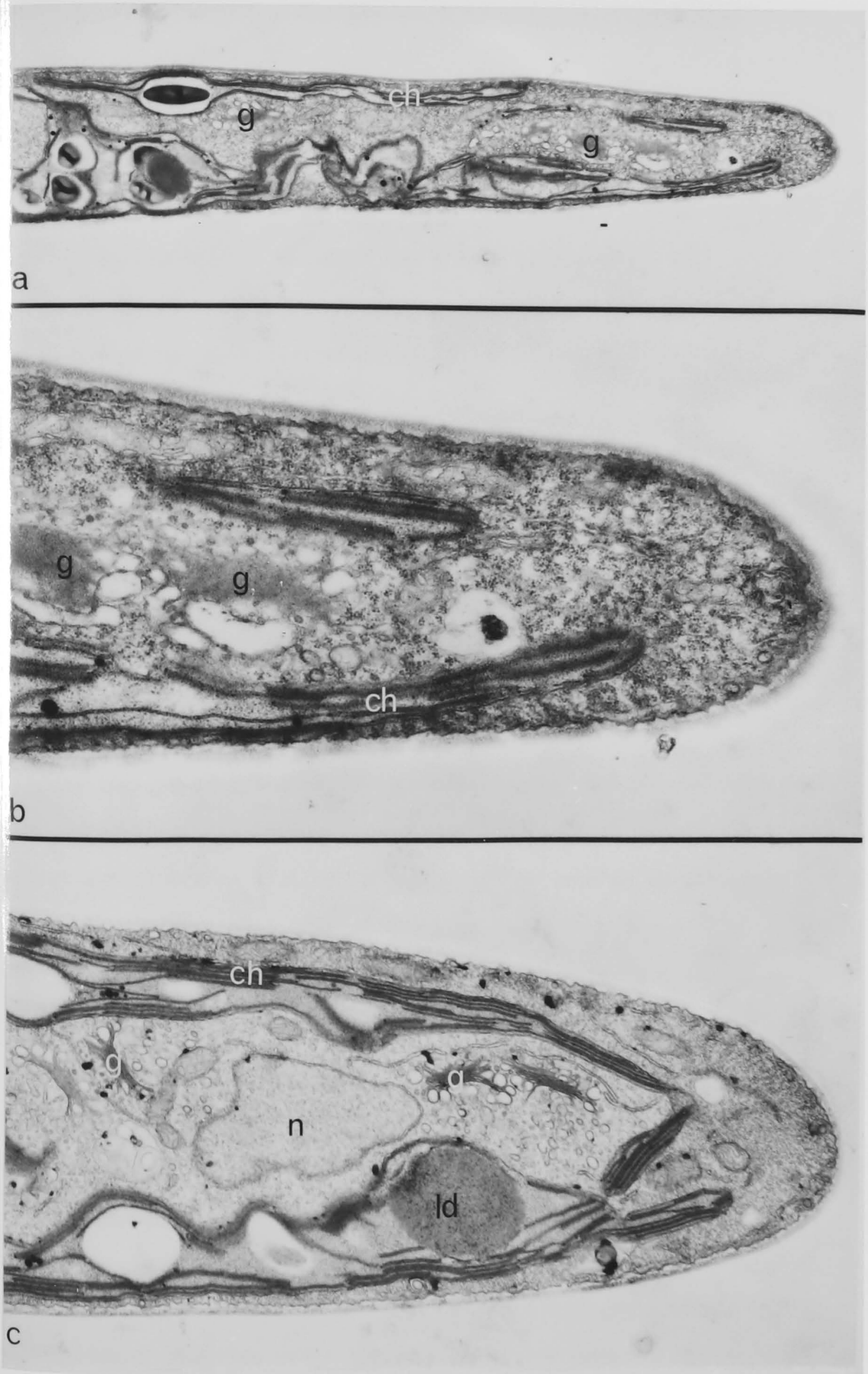

m
c
n
ch
g
g
a
m
ch
b
c

Fig. 2.26 Apex of developing branches in Stigeoclonium.

(a) Extensive vesicles and golgi bodies along the apical cell. (g, golgi; ch, chloroplast). X9,500.

(b) Dense cytoplasmic ribosomes and endoplasmic reticulum. (g, golgi; ch, chloroplast). X30,000.

(c) Active golgi bodies (g) and nucleus (n). Note the concentration of ribosomes at the apex. (ld, lipid droplets; ch, chloroplast). X16,000.


ch
g
g
a
g
g
ch
b
ch
g
d
n
ld
c

## 2.3.6 Normal Branching Patterns in Stigeoclonium

Pattern formation in Stigeoclonium is a dynamic process involving the interaction of cell division and branch initiation. Cell division increases the spacing between branches while branch initiation decreases the number of cells between branches.

The pattern of branching in Stigeoclonium has three aspects; the spacing of branches, the polarity of branches (defined on p.32) and the three-dimensional orientation of branches in relation to the axis of the main filament. These topics are dealt with in this section.

### Results

The spacing of branches is a phenotypically plastic character but under controlled culture conditions regular branching patterns can arise. The pattern of branch spacing in Stigeoclonium growing in nature (Fig.2.27) shows some similarity to that of the alga grown in culture (Fig. 2.28). Under both conditions the most frequent spacing between branches is six cells. The smaller peaks at spacings of 3 and 12 cells observed in culture were not shown in Stigeoclonium growing under natural conditions. The plants from nature were old and well established with both primary and secondary branches. By contrast, the plants in culture were young, actively growing filaments with only short primary branches (Fig. 2.28b and c).

In culture the polarity of most branches (92%) is apical although less strongly expressed under natural conditions (Table 2.6).

Fig. 2.27 Branch Pattern for Stigeoclonium growing in nature

Frequency distribution for 1000 cases.

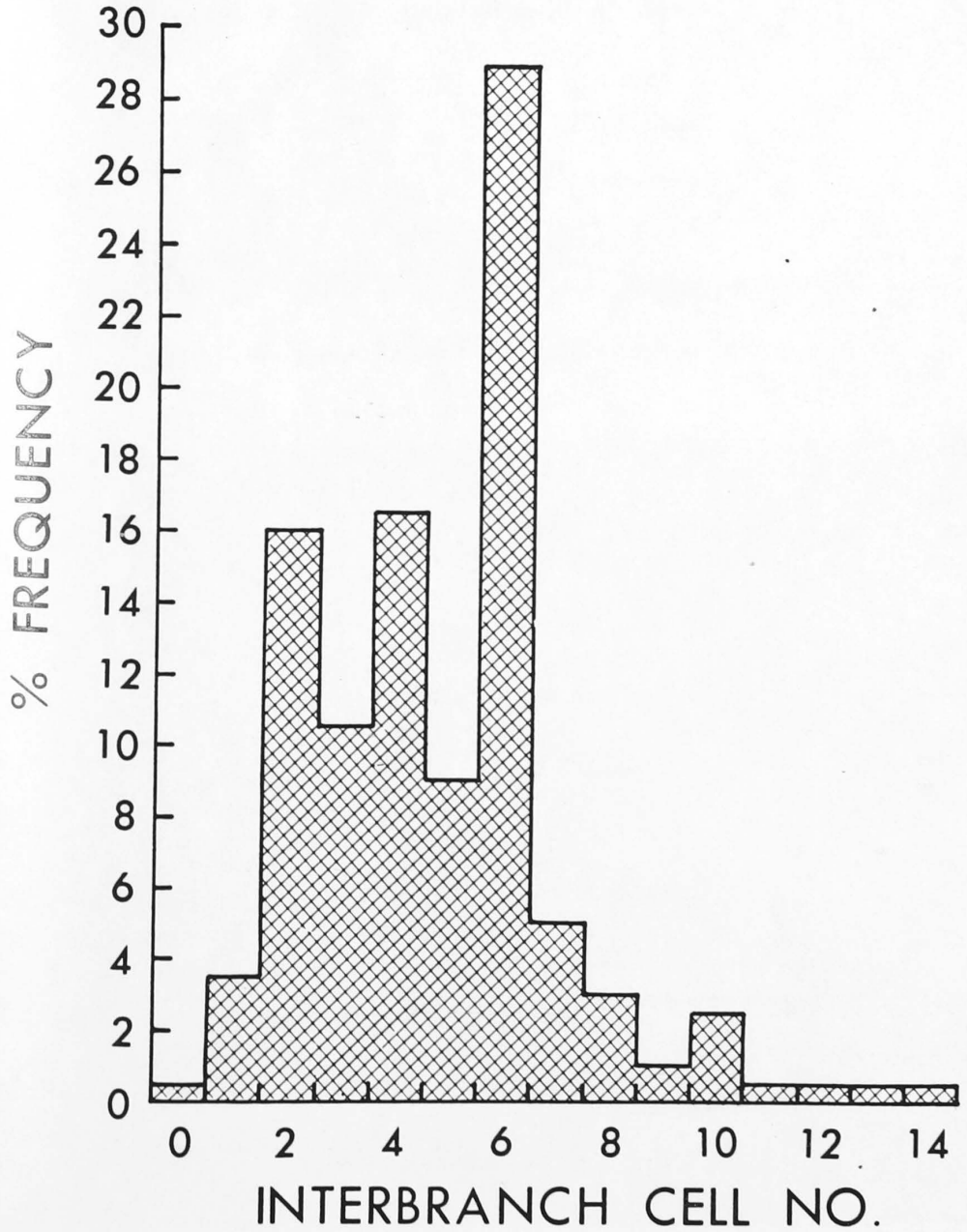

% FREQUENCY
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
INTERBRANCH CELL NO.

Fig. 2.28 Normal Branch Pattern Observed in Cultures

(a) Frequency distribution of the number of cells between branches for 1000 cases.

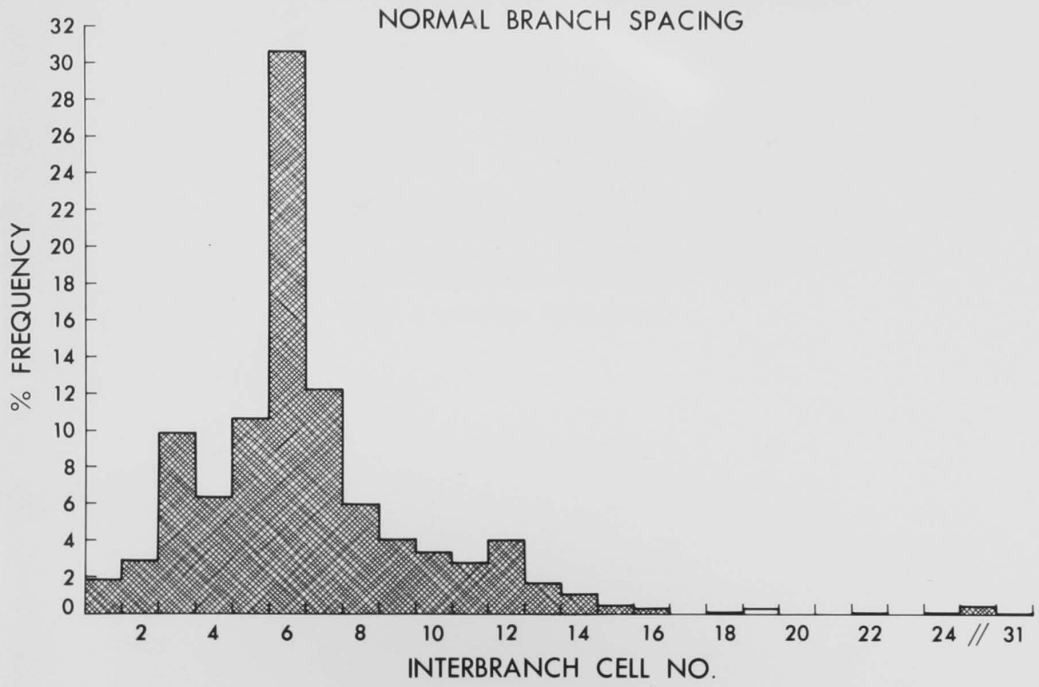
Mean 6.65 Variance 11.18

Skewness 1.97 Kurtosis 10.91

The central peak at the 6-cell spacing is far too sharp for a normal, double normal or log-normal distribution as determined by the MLP Programme.

(b) Regular three cell spacing between branches. Phase contrast X250.

(c) Regular six cell spacing between branches. Phase contrast X250.


NORMAL BRANCH SPACING
% FREQUENCY
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24 // 31
INTERBRANCH CELL NO.

a

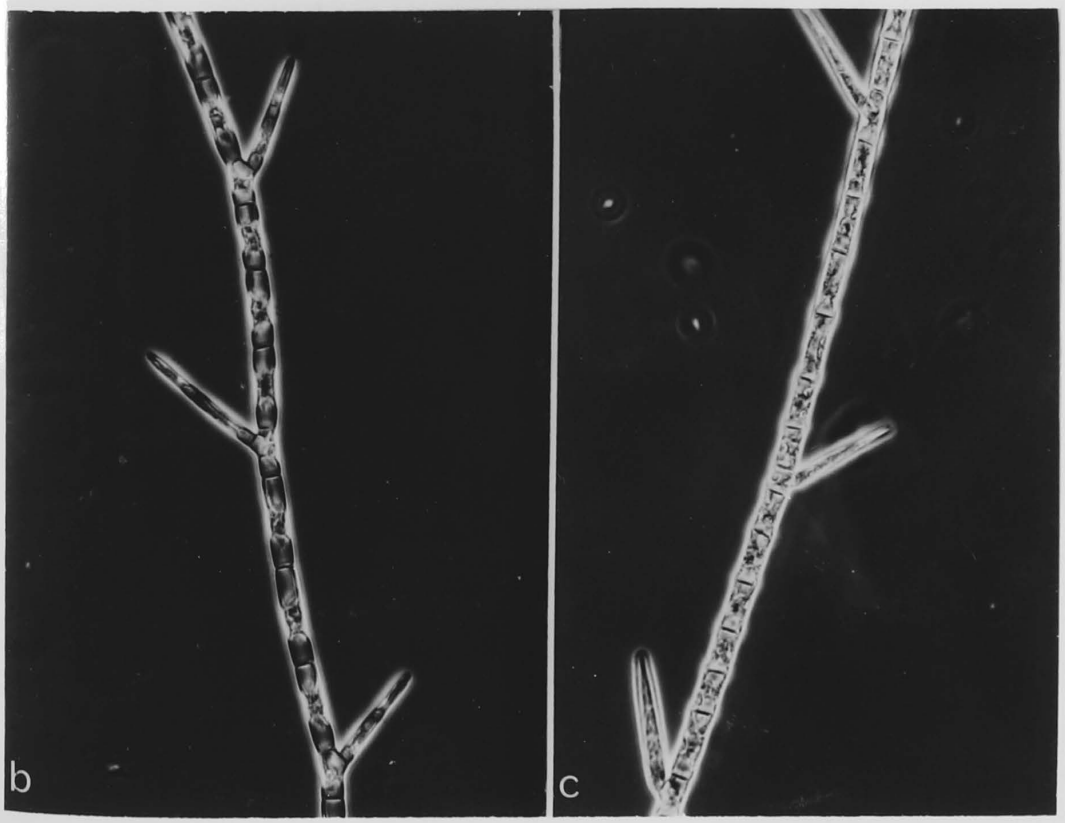

b
c

TABLE 2.6 POLARITY OF BRANCHING

IN CULTURE AND IN NATURE

(200 branches recorded)

| | Polarity | | |
|---|---|---|---|
| | *1 Apical | *2 central (apolar) | *3 basal |
| Culture | 92 | 3 | 5 |
| Nature | 81 | 6 | 13 |

Culture : Nature, Apical : remainder, 1:1

$\chi_1^2$ = 7.9 (significant at the 1% level)

*1. "Apical" means the branch originated from the apical end of an individual cell.

*2. "Apolar" means the branch originated from the centre of an individual cell.

*3. "Basal" means the branch originated from the base of an individual cell.

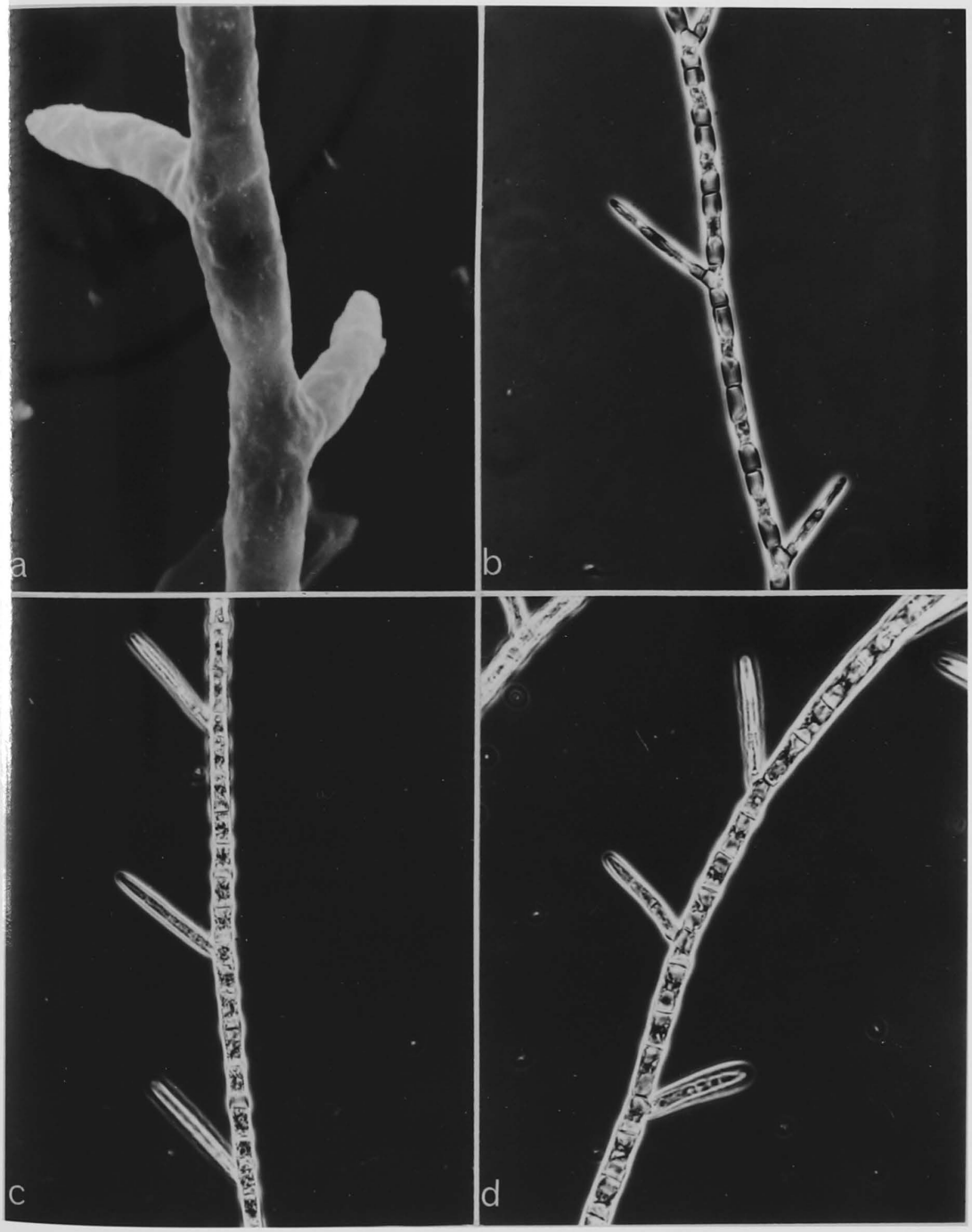
Fig. 2.29 Orientation of branches in Stigeoclonium.

(a) Scanning electron micrograph depicting the three-dimensional orientation of branches. X1,500.

(b) Light micrograph showing alternate branching. Phase contrast X250.

(c) Light micrograph of all the branches formed towards one side of the filament. Phase contrast X250.

(d) Light micrograph showing a combination of the orientation in (b) and (c). Phase contrast X250.


a
b
c
d

The orientation of branches is an extremely variable character. Although branching is often alternate (Fig. 2.29b) wide variations on this scheme of branching are common (Fig. 2.29c and d). It is extremely difficult to describe unequivocally the three-dimensional orientation of branches in the Stigeoclonium isolates, and it is reasonable to conclude that the orientation of branches is random.

## 2.4 General Discussion - Stigeoclonium as a Developmental System

The descriptive studies on the developmental morphology and life cycle of Stigeoclonium in this chapter were undertaken as a necessary prelude to the experimental studies on pattern formation and intercellular communication in the next chapter. These descriptive studies have highlighted the dynamic interplay of spatial and temporal factors in a catena of events which results in the branching patterns of Stigeoclonium. Some of the critical controls on the development of branching patterns in Stigeoclonium suggested by the descriptive studies are discussed below as a rationale for the experimental work in the next chapter.

### 2.4.1 Ultrastructure of Branch Development

The process of branch initiation as revealed by the ultrastructural studies presented here involved a co-ordinated movement of the nucleus and associated cytoplasm, a concentration of vesicles near the branch initial and an expansion of the vacuole at the end of the cell away from the branch initial.

#### Nuclear migration

Microfilaments and microtubules have been implicated in nuclear migration in a number of organisms (Hepler and Palevitz, 1974). The influence of the nucleus on the formation and orientation of side branches in the caulonema filaments of the moss Funaria hygrometrica

has been studied by Schmiedel and Schnepf (1979a). They concluded that the position of the nucleus determined the site of branch development and suggested that microtubules were involved in the movement of the nucleus since they increased during nuclear migration. By contrast the ultrastructural studies on Stigeoclonium presented in this chapter reveal no increase in the few microtubules associated with the migrating nucleus. Furthermore no microfilaments have been detected in the cytoplasm.

The dramatic expansion of one vacuole during branch initiation (Fig. 2.18b and c) suggests that the vacuole may provide the turgor pressure for moving the bulk cytoplasm during branch development. The role of the nucleus and the mechanism of its movement during branch initiation was investigated experimentally and is discussed in the next chapter.

## Nuclear-cytoplasmic interactions

The evolution of the nuclear membrane has resulted in uncoupling of transcription and translation, entailing the need to transfer information from the nucleus to the cytoplasm. It is well established that large quantities of macromolecules are exchanged between the nucleus and cytoplasm via the nuclear pores.

The nuclear membrane and particularly the membrane-pore complexes may control nuclear-cytoplasmic interactions by a selective transport of mRNA (Lichtenstein and Shapot, 1976). It has been suggested

that some of the central granules seen in the pores of the nuclear envelope may represent ribonucleoprotein particles in transit from the nucleus to the cytoplasm since marked reductions in the frequency of central granules in nuclear pores have been observed after treatment with actinomycin D (an inhibitor of DNA-dependent RNA synthesis), (Kessel, 1973). Animal cells undergoing active nuclear-cytoplasmic exchange often show discrete, densely staining bodies in the nucleus which appear to be transformed into a fibrous material as they move through the nuclear pores into the cytoplasm (Kessel, 1973). It is considered that the granules are composed of RNA and protein since they are not visible after digestion with pronase followed by RNase (Kessel, 1973).

The presence of discrete, densely staining bodies and nuclear pores with a central granule in the nucleus of Stigeoclonium (Fig. 2.8a) may suggest a marked nuclear-cytoplasmic exchange, possibly of RNA, during branch initiation. This is investigated further in the next chapter through the use of inhibitors of RNA and protein synthesis during branch initation.

## Vesicle Accumulation

The initiation of branches in Stigeoclonium involves a marked accumulation of vesicles at the site of initiation (Fig. 2.20b). Similar localized concentrations of vesicles have been documented in a number of tip growing systems including fungi (Grove and Bracker, 1970) root hairs (Bonnett and Newcomb, 1966) algal rhizoids (Sievers,1967) and pollen

tubes (Rosen, 1968). These apical vesicles are considered to be secretory vesicles, derived from the endomembrane system and they are thought to participate in tip growth by contributing new plasma-membrane, cell wall precursors, and/or wall softening materials to the expanding cell surface (Morré and Van Der Woude, 1974). The membranes for secretory vesicles of the golgi apparatus appear to be derived from endoplasmic reticulum or nuclear membrane, transformed during passage through the golgi apparatus for final incorporation into the plasma membrane. Northcote (1971) and Roberts (1974) have emphasized the crucial regulatory role of the golgi apparatus as a "one-way valve" in the membrane flow in plant cells. The major direction of membrane flow is from the nuclear envelope to the endoplasmic reticulum, to the golgi apparatus and via vesicles to the plasma-membrane. Either end of this pathway of membrane flow is reversible but the flow to and from the golgi apparatus is one way and therefore is a central control point. As the flow is one way, the golgi body is a polarised cell organelle with a forming face where membrane materials accumulate and a secretory face from which vesicles are released.

During branch initiation in Stigeoclonium the vesicles are transported and deposited at specific sites within the cell. Mollenhauer and Morré (1976) have suggested that the transport of similar vesicles in root tips is controlled by microfilaments.

Microtubules were present but rare, at the branch apex where active wall synthesis and deposition occurs in Stigeoclonium (Fig. 2.25 b and c). Microtubules are conspicuously absent from apices of other

tip-growing cells such as fern protonema (Stetler and De Maggio, 1972), fungal hyphae (Grove and Bracker, 1970), pollen tubes (Rosen, 1968) and root hairs (Newcomb and Bonnett, 1965). Ultrastructural studies of several algae including Pediastrum (Gawlik and Millington, 1969), Hydrodictyon (Marchant and Pickett-Heaps, 1972) and Cladophora (Robinson et al. 1972), have shown that microtubules are absent at the beginning of wall formation. The effect of inhibitors of microtubules and microfilaments on branch initiation in Stigeoclonium is discussed in the next chapter.

## 2.4.2 Cytokinesis

### Ultrastructure

The morphology of the cytokinetic apparatus has been used to distinguish two major classes of green algae, namely those with and those without a phycoplast (a complex system of microtubules oriented in the plane of cell division), (Pickett-Heaps, 1972; Pickett-Heaps and Marchant, 1972). This division of the green algae is supported by some biochemical studies (Frederick *et al*. 1973) and the morphology of motile cells (Marchant *et al*. 1973). The interzonal spindle of algae with phycoplasts collapses after telophase in contrast to the persistence of the interzonal spindle which keeps the daughter nuclei widely separated during cytokinesis of higher plants.

The phycoplast is regarded as indicating an evolutionary line distinct from the higher plants which use a phragmoplast in which the microtubules for cytokinesis are oriented perpendicular to the eventual plane of cell division. Thus *Stigeoclonium* which achieves cytokinesis by cell plate formation along a phycoplast system of microtubules (Fig. 2.15) is not regarded as giving rise to the progenitors of higher land plants but is the simplest branched filamentous alga of the Chlorophyceae (*sensu* Stewart and Mattox, 1975).

## Timing of Cytokinesis

The timing of cytokinesis in Stigeoclonium adds a temporal aspect to the spatial control of branching patterns. In an unbranched filament each cell divides once every twenty-four hours and is entrained to the light/dark cycle with most divisions occurring soon after the onset of light (Fig. 2.16, page 64).

The time of branch initiation can be related to the time of cytokinesis. In unbranched filaments, the first branch is often formed from a cell which divides at least one hour before neighbouring cells (Table 2.3, page 68).

In older filaments branch initiation normally occurs immediately after cytokinesis (Table 2.5, page 71). Meins (1975) has summarised evidence to support the hypothesis that the determination of cytodifferentiation in plants requires "a critical division, probably in the presence of specific inducers." However, cytokinesis per se may not be necessary for cytodifferentiation but rather a critical round of DNA synthesis in the presence of specific inducers may initiate the determination of cytodifferentiation in both animals (Holtzer, 1963) and plants (Foard, 1970 ; Dodds 1979).

The importance of the cell cycle and in particular, cytokinesis, for branch initiation in Stigeoclonium was investigated experimentally in the next chapter.

## The Cross Wall Segments

The cross wall areas formed during cytokinesis and which contain the plasmodesmata (discussed in the next section) may be of special significance in the development of Stigeoclonium.

Stewart et al. (1973) consider that the evolution of truly multicellular plants coincided with the evolution of plasmodesmata or other cytoplasmic continuity. The evolution of the filamentous habit in green algae is still controversial. Fritsch (1935) suggested that filaments evolved directly from motile unicells as reflected in the germination of zoospores of filamentous algae. By contrast some researchers believe it more likely that filaments developed from colonies of non-motile cells (see Smith, 1950). Another theory, proposed by Pickett-Heaps (1973) considers that "H-shaped" wall pieces such as occur in the green alga Microspora represent a primitive condition in the evolution of filaments. The H-pieces arise from two separate phases of wall secretion. During interphase, the interlocking H-pieces move apart to allow cell extension as a new cylindrical wall is secreted inside the H-shaped wall segments. During cytokinesis, the newly-formed cross wall transforms the cylinder into a H-shaped wall segment.

Similar H-shaped cross wall segments which are more resistant than lateral walls to enzymatic digestion were noted in Stigeoclonium during protoplast production (Fig. 2.10b) and zoospore release (Fig. 2.11d).

## 2.4.3 Plasmodesmata

In most cases the plasmodesmata of Stigeoclonium cells were found to be simple plasma-membrane lined pores of uniform diameter, but in some sections (Fig. 2.9b) a desmotubule apparently connected to the endoplasmic reticulum was observed, similar to that found by Floyd et al. (1972).

The presence of desmotubules in Stigeoclonium may have functional significance. A plasmodesmata containing a desmotubule allows two pathways for symplastic transport; one through the desmotubule from the endoplasmic reticulum cisternae of one cell to the next; or through the cytoplasmic annulus around the desmotubule (Robards, 1976). The desmotubule would allow a direct continuity of endoplasmic reticulum between adjacent cells and by linking the perinuclear spaces may provide a basis for intercellular nuclear interactions.

Stewart et al. (1973) considered that only those filamentous green algae with plasmodesmata have a significant differentiation of their thallus into specialized cells. The possible importance of plasmodesmata in controlling differentiation in Stigeoclonium is suggested by the occlusion and attenuation of plasmodesmata during zoospore formation (Fig.2.12). Similarly, during zoosporogenesis in Bulbochaete (Retallack and Butler, 1970) and Oedogonium (Pickett-Heaps, 1971) the plasmodesmata are blocked by the vesicle or hyaline layer which encloses the developing zoospore. Plasmodesmata may also have a role in maintaining the non-photosynthetic hair cells formed at the

ends of mature branches. Such a suggestion was made for hair cells in the filamentous green alga, Bulbochaete, by Fraser and Gunning (1973). The hair cells of Bulbochaete lack plastids but still produce abundant golgi vesicles which would require a supply of raw materials. It was presumed that these raw materials are transported to the hair cells via plasmodesmata from adjacent photosynthetic cells.

The plasmodesmata could represent the major barrier to the symplastic diffusion of molecules between Stigeoclonium cells.

The size of cells may determine the limitations on symplastic transport. In the giant cells of Chara, symplastic transport is not limited by plasmodesmatal resistance but by the velocity of symplastic streaming which delivers the solutes to the intercellular junctions (Walker, 1976). However in smaller cells (less than 40μm) the rate of symplastic transport may be determined by diffusion through the plasmodesmata (Tyree, 1970). Indeed the work of Worley (1968) on the small cortical cells and elongated fibre cells of stem internodes illustrates the effect of cell size on the limiting factors for transcellular transport of fluorescein. In both cell types plasmodesmata were similar and streaming was reversibly inhibited by dinitrophenol (DNP). However DNP treatment retarded the rate of cell-to-cell transport of fluorescein for the large fibre cells but had little effect on the small cortical cells. It was concluded that cell streaming limited the fast transport in elongated fibres but not in the small cortical fibres.

If one accepts Tyree's analysis (Tyree, 1970), the length of Stigeoclonium cells with a mode of 38μm (Fig. 2.13) would imply that in Stigeoclonium the symplastic diffusion of a morphogen may be controlled by plasmodesmatal resistance.

In simple, filamentous green algae such as Stigeoclonium which are bathed in an aqueous environment, plasmodesmata are not essential for the transport of nutrients since each cell could absorb its nutrients directly from the surrounding medium. Indeed green algae such as Spirogyra and Cladophora do not have plasmodesmata (see Marchant, 1976). However, the plasmodesmata of Stigeoclonium may be of importance in the development of branches by controlling both the spacing between branches as well as maintaining the highly differentiated, non-photosynthetic hair cells at the tips of mature branches.

In the next chapter the role of plasmodesmata in controlling branch patterns in Stigeoclonium was investigated by using techniques to disrupt symplastic transport between the interconnected cells of a filament.

## 2.4.4 Branch Patterns in Stigeoclonium

Local variations in nutrient supply and damage to the plants under natural conditions could contribute to the differences in branch patterns in nature compared with culture. The results indicate that there are optimal spacings between branches which are under coarse regulation by the developing plant. The apical polarity of the branches is strongly expressed in culture but significantly less so in nature. By contrast, the three-dimensional orientation of the emergence of a branch appears to be random.

Although the basic cellular events in development appear to be similar in plants and animals, plant development is more susceptible to modification by the external environment. Enclosure of its cells in more or less nondeformable cell walls results in a sessile plant that must be adapted to repair the ravages of predation and to environmental change. For higher plants "embryonic development continues throughout the life of the individual through the activity of the meristems, and it is continually sensitive to alteration by the external environment." (Sussex, 1972).

The branching pattern in Stigeoclonium has been shown to possess considerable phenotypic plasticity under different conditions of light and nutrients. Although Islam (1963) considered the pattern of branching in the erect filaments to be an important specific attribute, Cox and Bold (1966) found that the pattern of branching could be described only in broad and general terms because of the large degree of intraspecific

variation in the degree of branching. McLean and Benson-Evans (1977) also recorded an intraspecific variation in the degree of branching but found that the organism had fewer branches under high nutrient levels. They suggested that the frequency of branches of a population could be used to assess the degree of organic pollution in streams.

In general, Stigeoclonium has a denser branching pattern under nutrient-limiting conditions (Cox and Bold, 1966; McLean and Benson-Evans, 1977). In particular, low nitrate levels have been found to promote branching (Abbas and Godward, 1963; McLean and Benson-Evans, 1977). There may exist an optimal level of nitrogen for earlier workers found that high nitrate levels also promoted branching (Butcher, 1950; Uspenskaia, 1936). McLean and Benson-Evans (1977) have suggested that the surface area amplification that occurs through branching leads to more efficient absorption of a limiting ion from the water.

The orientation of branches in a variery of algae has been reported to be influenced by the direction of light. Under unilateral light, branches are formed on the side towards the light source (Klebs, 1896; Vischer, 1933; Islam, 1963). Klebs (1896) found that light from all sides induced branches in several directions. No attempt was made to repeat these unidirectional lighting experiments. It was hypothesized that the effect of unidirectional light may be due to relocation of elements of the cytoplasm, particularly the chloroplast, as is well known for Mougeotia (Haupt, 1972). However, light microscopic observations of cells of Stigeoclonium illuminated unilaterally using time-lapse cinematography revealed no directed movements of chloroplasts or other organelles such as are known in Mougeotia and other algae.

The process of pattern formation in Stigeoclonium can be related to the models discussed in Chapter 1.

The first branches are usually produced in six to seven day old cultures which contain approximately 30 to 70 cells. It has been noted for both animals (Wolpert, 1969) and the primary meristem of plants (Holder, 1979) that the size of the positional fields is often between 50 and 100 cells and less than 1 mm in length. Thus the primary branching patterns observed in this study of Stigeoclonium would have spatial dimensions similar to that of the classical fields of animals embryos.

The reaction-diffusion system of Turing (1952), especially its development by Martinez (1972), may provide a rationale for the random development of the first branch in a young filament of Stigeoclonium. Turing (1952) proposed that random disturbances caused a breakdown in an inherently unstable homogeneous equilibrium to produce a prepattern. In Stigeoclonium the manifestation of this initial disturbance may be a loss of synchrony of division in a cell in the young filament. Once this initial instability results in a pattern, continued cell division could result in a succession of instabilities and further differentiation (Martinez, 1972). This dynamic model of pattern formation which incorporates growth as an integral part of the pattern-forming process seems most applicable to Stigeoclonium in which the cells are continuously dividing.

Branching in Stigeoclonium is an example of a broad class of patterns called spacing patterns. Patterns which involve the spatial repetition of a similar element are often classified as spacing patterns.

Spacing patterns belong to a class of patterns whose development is thought to involve a considerable random element as well as an inhibitory mechanism preventing similar elements developing too close to each other (Wolpert, 1971). Examples of spacing patterns include the bristle patterns in the insect epidermis, stomatal patterns in leaves, phyllotaxis and the spacing of heterocysts in blue-green algae. These are discussed in detail at the end of Chapter 3.

The normal branching patterns in Stigeoclonium can best be explained if they are regarded as spacing patterns maintained by an inhibitory field around each branch.

The boundary regions and polarity of the developmental field in Stigeoclonium could be established at the time of germination of the zoospore and the formation of the erect filaments. The apical cell of the erect unbranched filament could be regarded as a source of a branch inhibitor while the basal cell may be the sink.

The presence of double branch points (Fig 2.19) could be explained if they are formed when the apical cell from the first branch has extended so that the concentration of inhibitor provided by intercellular transport to the basal cell is below the threshold level for branch inhibition. This is consistent with the observation that double branch points are only present in old cultures where the second branch is formed long after the initiation and development of the first branch. The occasional presence of branches adjacent to double branch points (Fig 2.19d) could also be explained on the same basis. Each cell may have the potential to form many branches but may be prevented from forming more than two branches by the combined inhibitory effect of the two apical cells.

The dynamic equilibrium between transverse cell division and branch initiation suggests that a mechanism may be involved in stabilizing an inhibitory gradient between branches. The gradient of the inhibitory field could be maintained by transport of the inhibitor via plasmodesmata between cells. Some aspects of this initial model are experimentally investigated and elaborated upon in the next chapter.

## CHAPTER 3 : EXPERIMENTAL STUDIES OF BRANCHING PATTERNS IN STIGEOCLONIUM.

## 3.1 INTRODUCTION

The transition from descriptive to experimental biology required the formulation of a theory of vital activity (Gasking, 1970). Before a subject can be considered an experimental science, the investigators must agree on a general theory to cover the subject which allows for an explanation of the known facts, provides guidance for further experimentation and is capable of modification in the light of new discoveries (Gasking, 1970).

The previous chapter described the developmental morphology and life cycle of Stigeoclonium. This chapter is concerned with experimental investigations into the process of pattern formation in Stigeoclonium. These experimental studies are based on the importance currently attributed to intercellular communication in theories of pattern formation. Most of the current theories of pattern formation incorporate some form of intercellular communication as a mechanism for establishing positional information.

Filaments of Stigeoclonium consist of a file of cells joined by intercellular connections, the plasmodesmata. These intercellular channels presumably provide at least a potentially functional continuity between all the cells of a filament. In this multicellular branched alga the pathways of communication for positional information are most likely to be the plasmodesmata. As Stigeoclonium inhabits flowing water apoplastic transport between cells does not seem likely as rapid loss by diffusion and dilution of extracellular products would appear inevitable.

In this chapter a number of techniques designed to disrupt intercellular communication between cells in a filament were employed to investigate the role of symplastic channels in the determination of branching patterns.

Plasmolysis was used to break and block plasmodesmata and thereby disrupt intercellular communication between the cells of a filament. Centrifugation, cutting and extirpation of cells with a laser microbeam were also used to the same end. Prolonged plasmolysis treatments were used to investigate the controls over polarity of branching.

A number of drug treatments was employed to investigate the mechanism of branch development. These treatments are critically discussed (including possible side effects) in their respective sections. Colchicine and cytochalasin B* were used to elucidate the role of microtubules and microfilaments respectively, in branch development. Binucleate cells were produced by caffeine treatment in order to investigate the role of the nucleus in branch development. The interaction between the cell cycle and branch development was investigated by using 5-aminouracil to synchronise cells to the final part of the S period of interphase. Actinomycin D was used to inhibit DNA-dependent synthesis of RNA during branch development while cycloheximide was used to inhibit protein synthesis on 80s ribosomes. Through the use of these drugs the relative timing and importance of these two events (RNA and protein synthesis) could be investigated during branch development after plasmolysis. The role of calcium in regulating intercellular communication and the position and polarity of branches was studied using the calcium ionophore A 23187 and the chelator EDTA.

* Cytochalasin B is widely used for studies of microfilament-mediated systems, and was chosen for use in the present work. However it does have certain side effects, see p.183.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Physical treatments

#### Plasmolysis treatments

All the temporary plasmolysis treatments were carried out at 1500 hours, two hours before the onset of the light period and branch development was recorded 24 hours later from random microscope fields as described in Chapter 2.

#### Effect of concentration of plasmolyticum

Cultures were treated for 30 minutes with different concentrations of mannitol made up in growth medium, washed once in medium and returned to fresh medium in the growth room. Mannitol was used at concentrations of 0.2M , 0.4M, and 0.6M.

#### Effect of the period of treatment with the plasmolyticum

Cultures were treated with 0.6M mannitol in growth medium for periods of 1, 5, 10, 15 and 30 minutes.

#### Standard Plasmolysis Treatment

Cultures were plasmolysed for 30 minutes by replacing the growth medium with 0.6M mannitol dissolved in growth medium. This and the treatments listed above was carried out under green lights in the growth room.

## Prolonged Plasmolysis Treatments

A standard plasmolysis treatment was followed by culturing for 48 hours in growth medium for controls, and 0.2M, 0.4M and 0.6M mannitol dissolved in growth medium for the experimental treatments. Polarity of branches was recorded for 200 cells not associated with dead cells by recording random microscope fields for each treatment.

Since branch development did not occur during the 48 hour treatment with 0.6M mannitol in growth medium, plants exposed to this treatment were subsequently transferred to growth medium, and the polarity of branch development was recorded 48 hours later.

## Plasmolysis during the cell cycle

Cultures were given a standard plasmolysis treatment at
1500 hours (2 hours before the onset of light),
1700 hours (the start of the light period),
1900 hours (2 hours after the onset of light),
and 2100 hours (4 hours after the onset of light).

The cultures were then stained in Calcofluor for fifteen minutes and washed with two changes of growth medium. Branch development recorded exactly 24 hours after the respective time of plasmolysis.

## Centrifugation

Coverslips were cut to fit 10 ml centrifuge tubes. Cultures were then grown on these coverslips in Petri dishes. The coverslips on which plants had become attached were centrifuged at 1000 Xg for 30 minutes using an M.S.E. bench centrifuge and then transferred back to fresh growth medium.

## Surgical Experiments

Cultures were placed on fresh dental wax and cut into sections of randomly varying length using razor blades. Branch and rhizoid development were recorded photographically 24-48 hours after cutting and further growth in fresh medium.

## Laser Studies

A laser microbeam was used to study the effect of cell death on branch development within filaments of Stigeoclonium. The system described by Marchant (1978) comprising a flashlamp pumped dye laser coupled with a Zeiss photomicroscope produces a spot size of 3-5 μm. Individual plants were removed by using glass Pasteur pipettes. The plants were then mounted in culture medium on a microscope slide and after adding a coverslip were examined under a Zeiss photomicroscope. After establishing the aiming mark with a red blood cell smear, individual cells were lased. The power output of the laser was in the range of 60-160 m Joules. By using neutral density filters, 3% of this output reached the cells giving a power input to the cells of between 1.8 and 18 m Joules. Each experiment was undertaken ten times.

## 3.2.2 Drug Treatments

For the quantitative data from these drug treatments, branching was observed in 200 cells selected within microscope fields (X40 objective, 1.25 Optivar, X10 ocular) at random. Calcofluor staining (see Section 3.2.3) was used before treatments to ensure that only those branches formed during or after treatment were recorded.

Colchicine - Colchicine (Sigma Chemical Co., U.S.A.) was used at a concentration of 0.2% dissolved in culture medium. The drug was applied to cultures after a standard plasmolysis treatment at 1500 hours. The cultures were treated continuously with the drug for 24 hours and then branch polarity was recorded. Controls were plasmolysed and then placed in fresh culture medium.

Cytochalasin B - Cytochalasin B (Sigma Chemical Co., U.S.A.) was used at a concentration of 50 µg/ml. Stock solutions were prepared in dimethylsulphoxide (DMSO); on dilution with growth medium the concentration of DMSO became 0.5%. The drug was applied to cultures after a standard plasmolysis treatment at 1500 hours. The cultures were treated continuously with the drug for 24 hours and then branch polarity was recorded. Controls were plasmolysed at the same time and then placed in 0.5% DMSO in culture medium.

Caffeine - Caffeine (B.D.H. Biochemicals, Victoria, Australia) was used at a concentration of 0.1% in culture medium for 24-48 hours. Cultures were then given a standard plasmolysis treatment and placed in fresh culture medium. The branch development was recorded photographically 24 hours after plasmolysis.

5-Aminouracil - 5-aminouracil (Sigma Chemical Co., U.S.A.) was used at a concentration of 500 ppm dissolved in culture medium for 24 hours to synchronise cells at the S-phase of the cell cycle. Cultures that had been exposed to the drug were given a standard plasmolytic treatment at 1500 hours and then returned to normal growth medium. The control cultures were returned to normal growth medium after the 24 hour drug treatment without any plasmolysis. Other control cultures were given the standard plasmolysis treatment and returned to normal growth medium without any treatment with 5-aminouracil. Branch frequency was recorded 24 hours after plasmolysis.

Cycloheximide - Cycloheximide (Sigma Chemical Co., U.S.A.) was used at a concentration of 25 µg/ml in culture medium. The cultures were subjected to a standard plasmolysis treatment at 0900 hours and then placed into solutions containing the drug at 0, 3, 6, 9 and 12 hours after plasmolysis. Cultures were stained with Calcofluor immediately after plasmolysis. The frequency of branching was determined 24 hours after plasmolysis. Controls consisted of plasmolysed cells returned to culture medium and a continuous 24 hour treatment with the drug. Cultures treated with cycloheximide after plasmolysis were also washed with two changes of fresh culture media to study the recovery after washing out the drug.

Actinomycin D - Actinomycin D (Grade 1 from Sigma Chemical Co., U.S.A.) was used at a concentration of 100 µg/ml in culture medium. The actinomycin D was dissolved in acetone and subsequently diluted with culture medium so that the final concentration of acetone in the medium

did not exceed 0.05%. Controls also contained this concentration of acetone. This drug treatment was run in parallel to that for cycloheximide and the experimental design was identical to that detailed above for cycloheximide.

### 3.2.3 Microscopy

The standard light and electron microscope procedures detailed in Chapter 2 were also used in these experimental studies and will not be repeated here.

#### Fluorescence microscopy

Calcofluor staining - Calcofluor was used at a concentration of 0.1% in culture medium. The cultures were stained for 10 to 15 minutes and then washed twice in growth medium. The Calcofluor staining was detected by using a Zeiss ultraviolet fluorescence microscope with exciter filter UG1 (transmits 300-400 nm with 365 nm peak) and barrier filter number 41 (transmits above 410 nm). With this filter combination the Calcofluor was seen to bind to the cell wall, where it fluoresced strongly with a light blue colour as described by Hughes and McCully (1975). Calcofluor does not normally cross the plasma-membrane and stain the cytoplasmic components of undamaged cells. It was used in the experiments described in this section to detect cells whose plasma-membrane had been damaged as well as to detect cells which had divided or produced branches (as described in Chapter 2) during or after experimental treatment.

Aniline blue staining - Aniline blue (Gurr, water soluble) was used at a concentration of 0.05% in 0.06M $K_2HPO_2$(pH 9.1) to locate callose deposition. The cultures were stained with aniline blue for 10 to 15 minutes and then examined using exciter filter BG 12 (transmits 325-500 nm with peak at 400 nm) and barrier filter number 53 (transmits above 530 nm). Control treatments consisting of 0.06M $K_2HPO_4$(pH 9.1) were found not to induce callose fluorescence. The specificity of aniline blue staining is discussed in section 3.4.3.

Electron Microscopy

The standard techniques of electron microscopy detailed in Chapter 2 were employed to observe the effects of plasmolysis on plasmodesmata. Filaments were fixed and embedded after 24 hours in growth medium, following a standard plasmolysis treatment for 30 minutes in 0.6M mannitol in growth medium.

### 3.2.4 Studies on intercellular communication in Stigeoclonium.

Callose deposition during plasmolysis

Cultures were plasmolysed with mannitol then stained with aniline blue as described above and then examined with the fluorescence microscope for callose deposition. Controls consisted of cultures in the phosphate buffer used to dissolve the aniline blue as described in section 3.2.3.

## Calcium and Callose interactions

Cultures were washed in calcium-free Bold's Basal Medium (BBM) and then placed in $10^{-2}$ M EDTA in calcium-free BBM for 1 hour. EDTA was readily soluble in growth medium, and the pH was adjusted to normal (pH 6.8). After treatment with EDTA the cultures were plasmolysed for 30 minutes in 0.6M mannitol made up in calcium-free BBM. Cultures were then immediately stained with aniline blue and examined for callose deposits as detailed in section 3.2.3. The control cultures were plasmolysed without any EDTA treatment while some cultures treated with EDTA were transferred to normal BBM which contains $10^{-3}$ M calcium.

## Studies using X-ray microanalysis

An EDAX model 707A was used on a Jeol 100B Electron Microscope with an ASID (Attachment Scanning Image Device). Counts were made for one hundred and two hundred seconds on areas near the cross walls of plasmolysed Stigeoclonium cells. The material was fixed and embedded using the standard techniques detailed in Chapter 2. However in this case the material was fixed in glutaraldehyde containing 0.6M mannitol. Control tissues were fixed in glutaraldehyde without mannitol.

## Calcium ionophore studies

The calcium ionophore, A 23187 (a gift from Lilly Ltd.) was used at a concentration of $5 \times 10^{-5}$ M. It was dissolved initially in ethanol and sonicated for 30 - 60 minutes. For experimental use the ionophore was

diluted in growth medium -(BBM)so that the final concentration of ethanol was not greater than 1%. Cultures were treated for forty-eight hours with the calcium ionophore and then placed in fresh culture medium(BBM)containing $10^{-3}$ M calcium. Branch patterns were recorded during & after treatment. Control cultures were treated with 1% ethanol in culture medium(BBM). All the cultures were stained with aniline blue to detect callose after treatments.

Growth studies in calcium-free Bold's Basal Medium

Cultures were grown in a calcium-free BBM for 7-10 days and branching patterns recorded photographically.

Fluorescein diacetate (FDA) Transport Studies

Fluorescein diacetate (FDA) was used at a concentration of 0.02% in growth medium.

Following uptake by a cell the fluorescein diacetate is converted in the cytoplasm to the fluorescent dyestuff fluorescein by esterase. The kinetics of this conversion have been studied in detail by Sontag (1977). The symplastic transport of fluorescein was followed microscopically using exciter filter BG 12 (transmits 325-500 nm with peak at 400 nm) and barrier filter number 47 (transmits above 470 nm). Fresh filaments were removed from culture and laid on a microscope slide which had two thin strips of Valap (a 1:1:1 mixture of Vaseline:lanoline:paraffin) so as to isolate a short section of the filament sealed off at both ends. Fluorescein diacetate was added to the left hand section of the filament, the sealed section in the middle was used for experimental treatments

such as plasmolysis and the transport through the filament was monitored by observing the fluorescence of the right hand section. As a check for the integrity of the Valap seal, Calcofluor (0.1% in culture medium) was added to the left hand segment and its leakage monitored in both the experimental chamber in the centre and the right hand section. Calcofluor does not penetrate undamaged cells and is therefore not transported symplastically along the filament. The transport of the fluorescent dyes was observed after a coverslip was gently placed on top of the filament.

## Intercellular electrical coupling

The technique of current injection described by Drake et al. (1978) was used in an attempt to monitor the intercellular electrical coupling between Stigeoclonium cells. Filaments of Stigeoclonium were embedded in cooled agar and then mounted in a perspex chamber and perfused with culture medium during these electrophysiological studies.

## 3.3 RESULTS

### 1. Effect of concentration of plasmolyticum on branch frequency

The frequency of branching is increased when the concentration of osmoticum is 0.4M or higher (Table 3.1). The most marked increase in branch frequency was obtained in cells treated for 30 minutes with 0.6M mannitol, which induced full plasmolysis, while the treatment at less than incipient plasmolysis (0.2M) had no apparent effect. Thus initiation of branching is dependent on the concentration of osmoticum for a 30 minute treatment.

### 2. Time-course of plasmolysis

Similar but less consistent branching responses were obtained by treating filaments with osmoticum (0.6M mannitol) for brief periods (1-5 minutes) which did not result in a retraction of the protoplast observable by light microscopy (Table 3.2). Observations with the light microscope revealed no polarity in the plasmolysis of cells. In some cases the protoplasts of two adjacent cells were both slightly retracted from the septum after 30 minutes in 0.6M mannitol (Fig. 3.1a) while in other cases there was a complete retraction of one protoplast and only a partial retraction of the other (Fig. 3.1b). Prolonged treatment of 1 hour or 3 hours in 0.6M mannitol resulted in marked retractions of all protoplasts (Fig. 3.1c and d).

These results (Table 3.2) show that brief osmotic treatments, insufficient to produce observable plasmolysis themselves, can induce

TABLE 3.1 THE EFFECT OF CONCENTRATION OF PLASMOLYTICUM ON BRANCH FREQUENCY

| Concentration of Mannitol | % Plasmolysed cells after 30 mins. (200 cells) | Branching frequency (%). (200 cells) |
|---|---|---|
| 0 (control - growth medium) | 0 | 17 |
| 0.2M | 0 | 19 |
| 0.4M | 11 | 38 |
| 0.6M | 73 | 61 |

TABLE 3.2 THE EFFECT OF TIME OF TREATMENT WITH 0.6M MANNITOL ON BRANCH FREQUENCY

| Treatment Time (mins.) | Branch Frequency (%) (200 cells) |
|---|---|
| 0 | 21 |
| 1 | 38 |
| 5 | 43 |
| 10 | 42 |
| 15 | 48 |
| 30 | 59 |

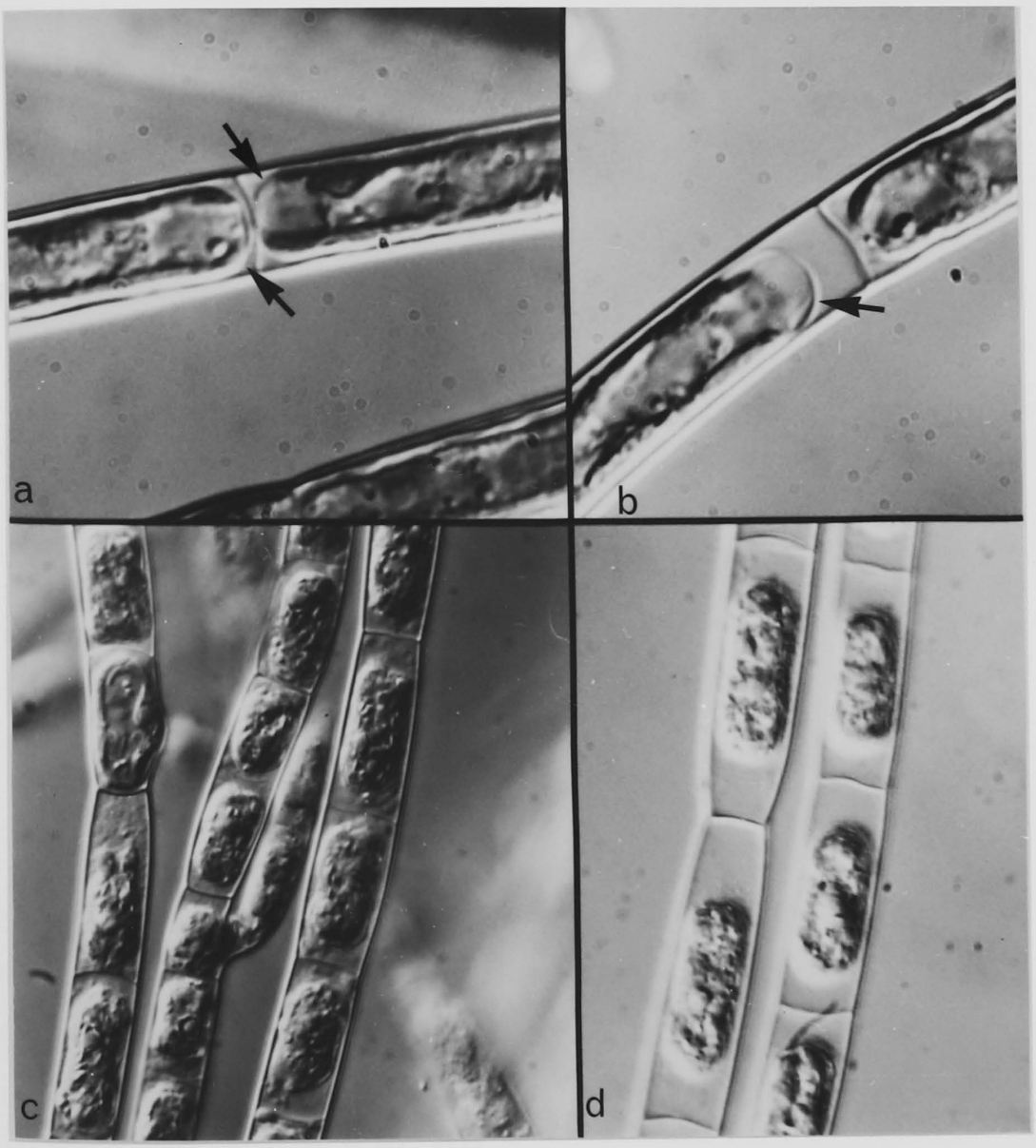
Fig. 3.1 Plasmolysis of Stigeoclonium cells

(a) Slight retraction of both protoplasts (arrows) from the septum after 30 minutes in 0.6M mannitol. Nomarski X1,200.

(b) Complete retraction of one protoplast (arrowed) from the septum and only partial retraction of the other after 30 minutes in 0.6M mannitol. Nomarski X1,200.

(c) Retraction of protoplasts after one hour in 0.6M mannitol. Nomarski X850.

(d) Marked retraction of protoplasts after 3 hours in 0.6M mannitol. Nomarski X850.


a
b
c
d

branching in Stigeoclonium. This suggests that small osmotic changes sufficient to close or block plasmodesmatal channels but not to break plasmodesmata are able to induce a branching response presumably by temporary physiological isolation of cells.

Thus, as established in the previous section, branch initiation is dependent on the concentration of osmoticum and on the time of treatment at the 0·6M level (see Table 3·2).

3. The effect of temporary plasmolysis on branch formation and branching patterns in Stigeoclonium.

Temporary plasmolysis for 30 minutes with 0.6M mannitol induced a single branch to arise from the majority of cells and cell division during the subsequent twenty-four hours resulted in a one cell spacing between the branches (Fig. 3.2).

In a few cases a gradient in rate of branch initiation was observed; cells closer to the base developed faster than those near the apex (Fig. 3.2b). However the majority of branches developed at approximately the same rate.

In cultures observed a few days after plasmolysis the cell between the branches has remained undivided but the new branch has developed normally (Fig. 3.2d). Thus the six-cell spacing of branches common in unplasmolysed cultures is not restored by divisions between the branches induced by temporary plasmolysis.

Fig. 3.2 Branch Pattern after temporary plasmolysis

(a) Frequency distribution of the number of cells between branches for 1000 cases.

(b) Branch development 24 hours after plasmolysis. Nomarski X700.

(c) and (e)

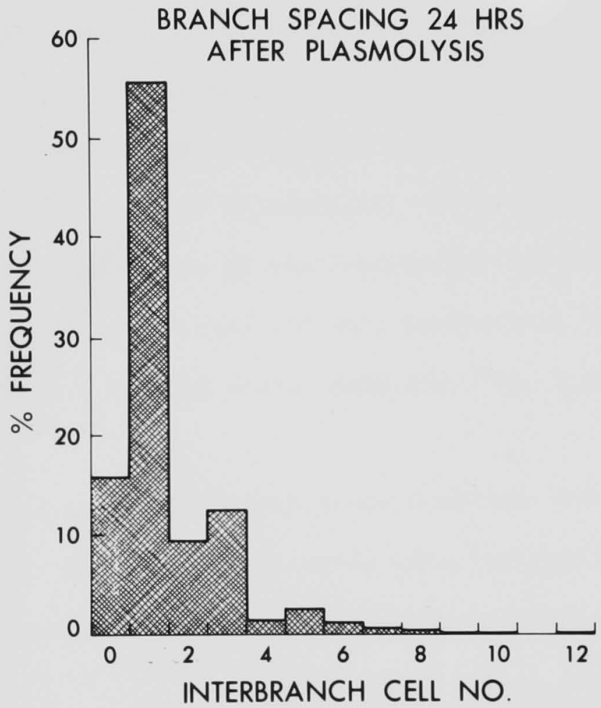
Cells plasmolysed, stained with Calcofluor, and then examined 24 hours later with the fluorescence microscope. The division of each cell during the 24 hour period is clearly illustrated as a faintly fluorescing new septum* (arrows) between the bright Calcofluor fluorescence of the old septa (arrowheads).

(c) Phase contrast and fluorescence microscopy X250.

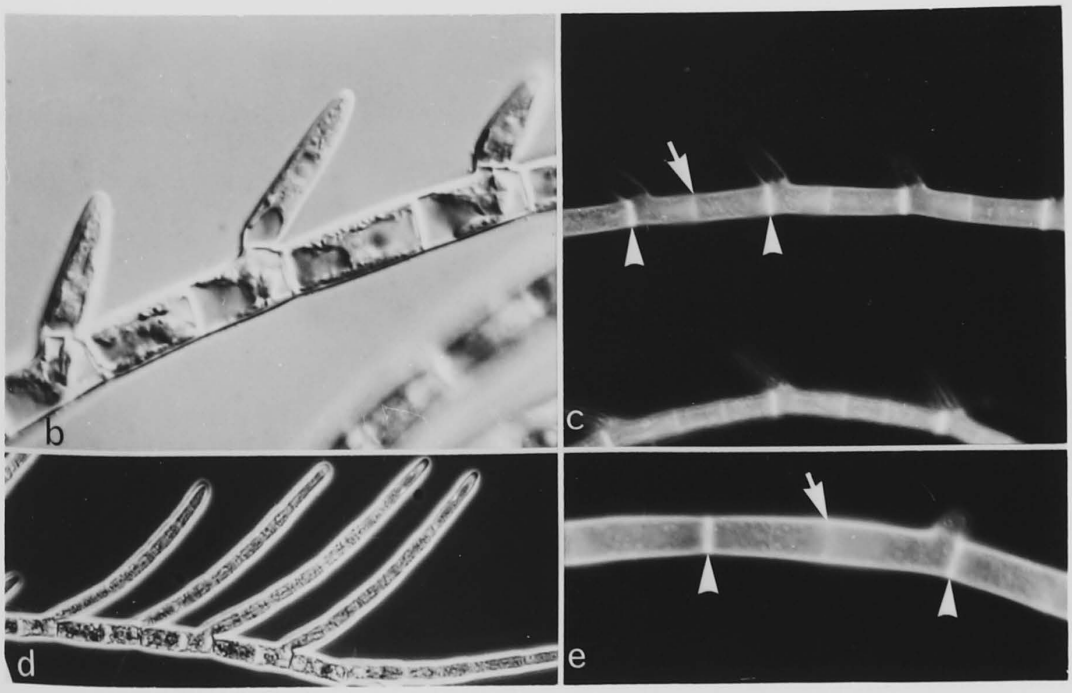
(e) Phase contrast and fluorescence microscopy X350.

(d) Three days after temporary plasmolysis the branches have grown considerably but the interbranch cells have not divided so that a one cell spacing is maintained. Phase contrast X200.

* The faint fluorescence of the new septa may be due to residual strain.


BRANCH SPACING 24 HRS
AFTER PLASMOLYSIS
% FREQUENCY
60
50
40
30
20
10
0
0
2
4
6
8
10
12
INTERBRANCH CELL NO.

a


b
c
d
e

The polarity of most branches is apical. In a small percentage of cases, branching polarity was reversed (Fig. 3.3). Branches with reversed polarity commonly arose from cells adjacent to cells that had been killed as a result of plasmolysis. These dead cells could most readily be detected by use of the fluorescent dye Calcofluor which is normally excluded by the cell and only accumulates in the cytoplasm of those cells with a damaged plasma membrane (Fig. 3.3c and d).

In most cases the branch arose from that end of the cell nearer the dead cell. When individual cells were isolated between dead cells they appear equally likely to give rise to branches at either end of the cell.

A further observation on polarity of branch formation after plasmolysis was made using cells which were beginning to form zoospores. These cells can be readily detected in culture because they are much smaller than the neighbouring cells and often have a denser cytoplasm. After plasmolysis the polarity of branch development from these cells appears to be random (Fig. 3.4) and there seems to be no control over the polarity of branching even though cell death has not occurred.

Fig. 3.3 Changes in branch polarity after cell death caused by plasmolysis.

(a) and (b)

Branches of opposing polarity are shown adjacent to dead cells (arrows). (a) Phase contrast X350, (b) Phase contrast X500.

(c) and (d)

Dead cells (arrows) associated with branch regeneration can be most readily detected after Calcofluor staining.

(c) Phase contrast and fluorescence microscopy X600.

(d) Phase contrast and fluorescence microscopy X450.

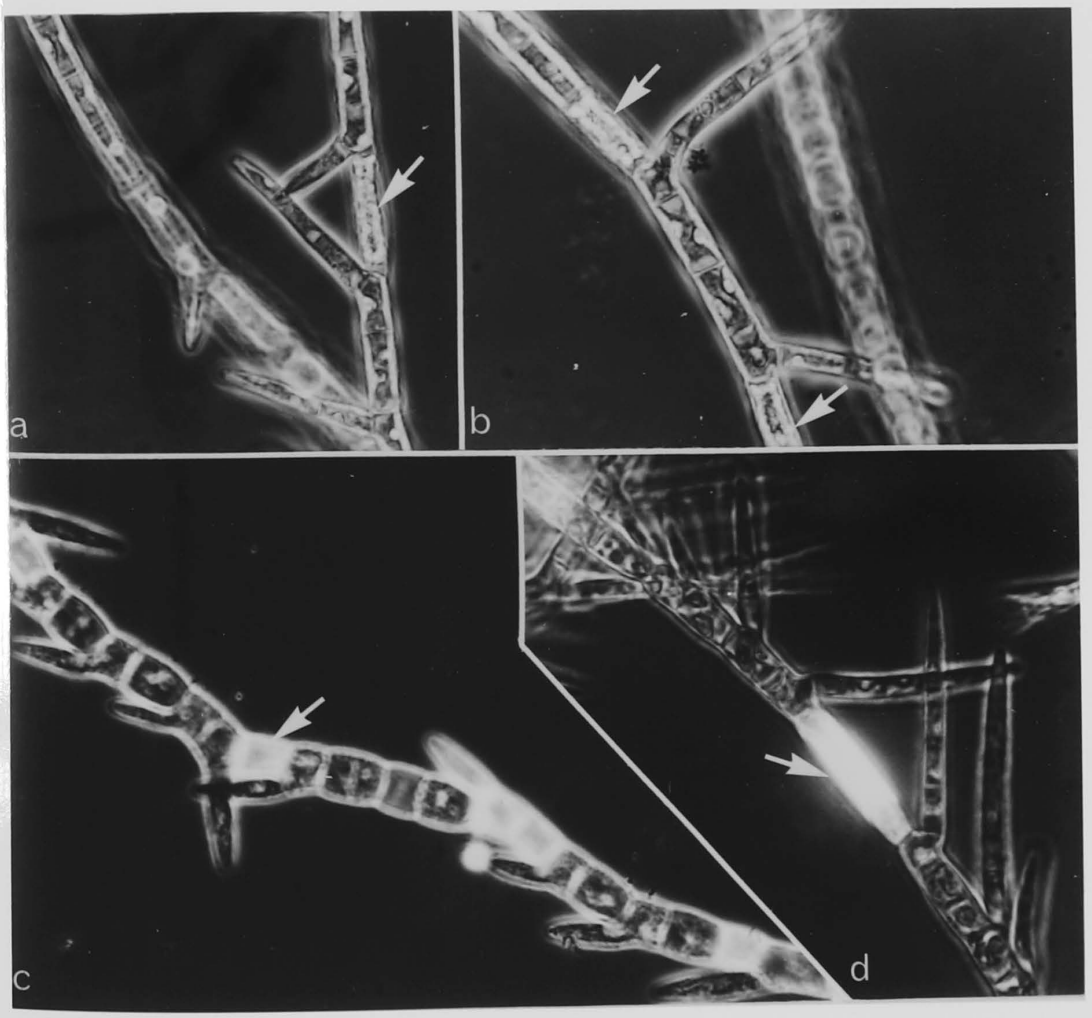

a
b
c
d

Fig. 3.4

Development of branches after plasmolysis of mature cells, characteristic of cells about to undergo zoosporogenesis. Note the variability in polarity of the branches.
Phase contrast X750.

## 4. The effect of prolonged plasmolysis treatments on branch patterns in Stigeoclonium.

If plants are kept for prolonged periods (48 hours) in 0.4M mannitol or 0.2M mannitol in culture medium after plasmolysis there are changes in the branch polarity as compared with those returned to normal medium immediately after plasmolysis (Fig. 3.5). In 0.4M mannitol there is a large increase in the number of branches which are apolar, those which extend from the middle of each cell. If cells are kept in 0.6M mannitol for 48 hours and then returned to normal growth medium the numbers of polar branches growing towards the apex and those directed towards the base is approximately equal (Fig 3.5). In this case, polarity is expressed in the sense that branches appear at the cell ends, but the branches are formed in approximately equal numbers towards the base and apex of the cell, suggesting that control of the polarity of the cell is lost or relaxed during prolonged plasmolysis.

During ordinary polar branch formation only one large vacuole is seen near the basal wall of the cell. The normal cell has vacuoles at either end of the cell (see Chapter 2). When maintained in 0.4M mannitol there are two vacuoles that remain at either end of the cell and the branches are formed between these two vacuoles (Fig. 3.6). Also during treatment with 0.4M mannitol the nucleus and associated cytoplasm does not move to one end of the cell. This contrasts with the initiation of a normal polar branch when the nucleus is positioned at the initiating end of the cell. Occasionally unusual branch formation was observed to follow incipient plasmolysis. Sometimes a weakly polar branch was formed at an angle greater than $45^{o}$ to the main filament but

Fig. 3.5 Changes in polarity of branches observed 48 hrs. after various plasmolytic treatments.

(200 branches recorded for each treatment)

(a) Branch polarity (in growth medium) after 30 minutes plasmolysis in 0.6M mannitol.

(b) Branch polarity (in 0.2M mannitol in growth medium) after 30 minutes plasmolysis in 0.6M mannitol.

(c) Branch polarity (in 0.4M mannitol in growth medium) after 30 minutes plasmolysis in 0.6M mannitol.

(d) Branch polarity upon returning to medium after prolonged plasmolysis (48 hours) with 0.6M mannitol in growth medium.

A:B, 0:-, 1:1

$X_1^2$ = 17.1 (significant at the 1% level)

A,B and C follow a trend with a major shift from the + to o category.

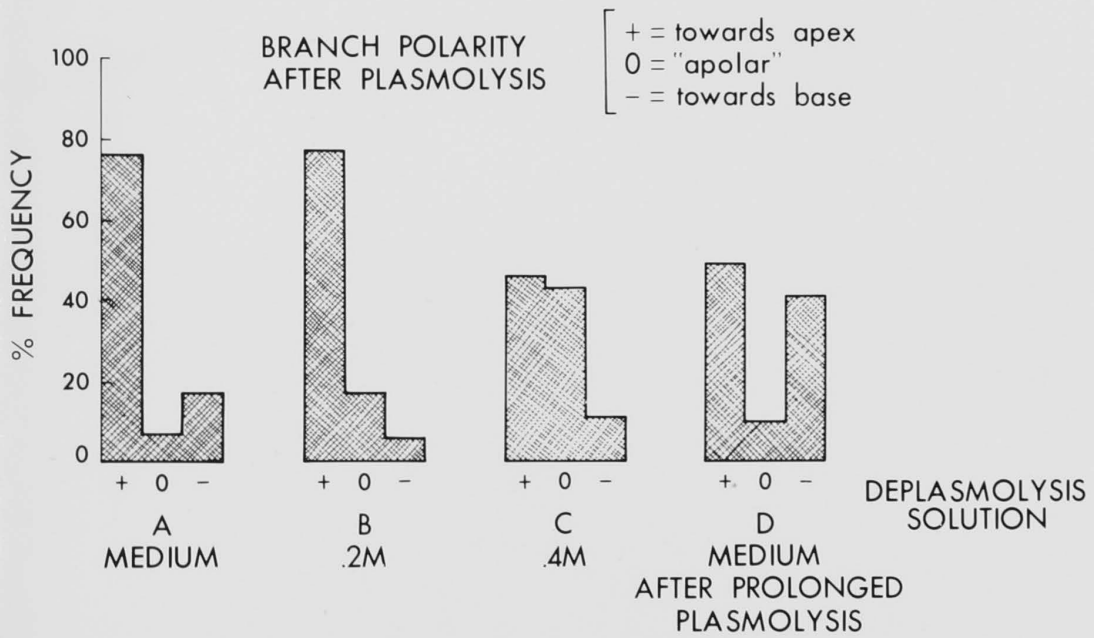

BRANCH POLARITY
AFTER PLASMOLYSIS
+ = towards apex
0 = "apolar"
− = towards base
% FREQUENCY
100
80
60
40
20
0
+ 0 −
A
MEDIUM
B
.2M
C
.4M
D
MEDIUM
AFTER PROLONGED
PLASMOLYSIS
DEPLASMOLYSIS
SOLUTION

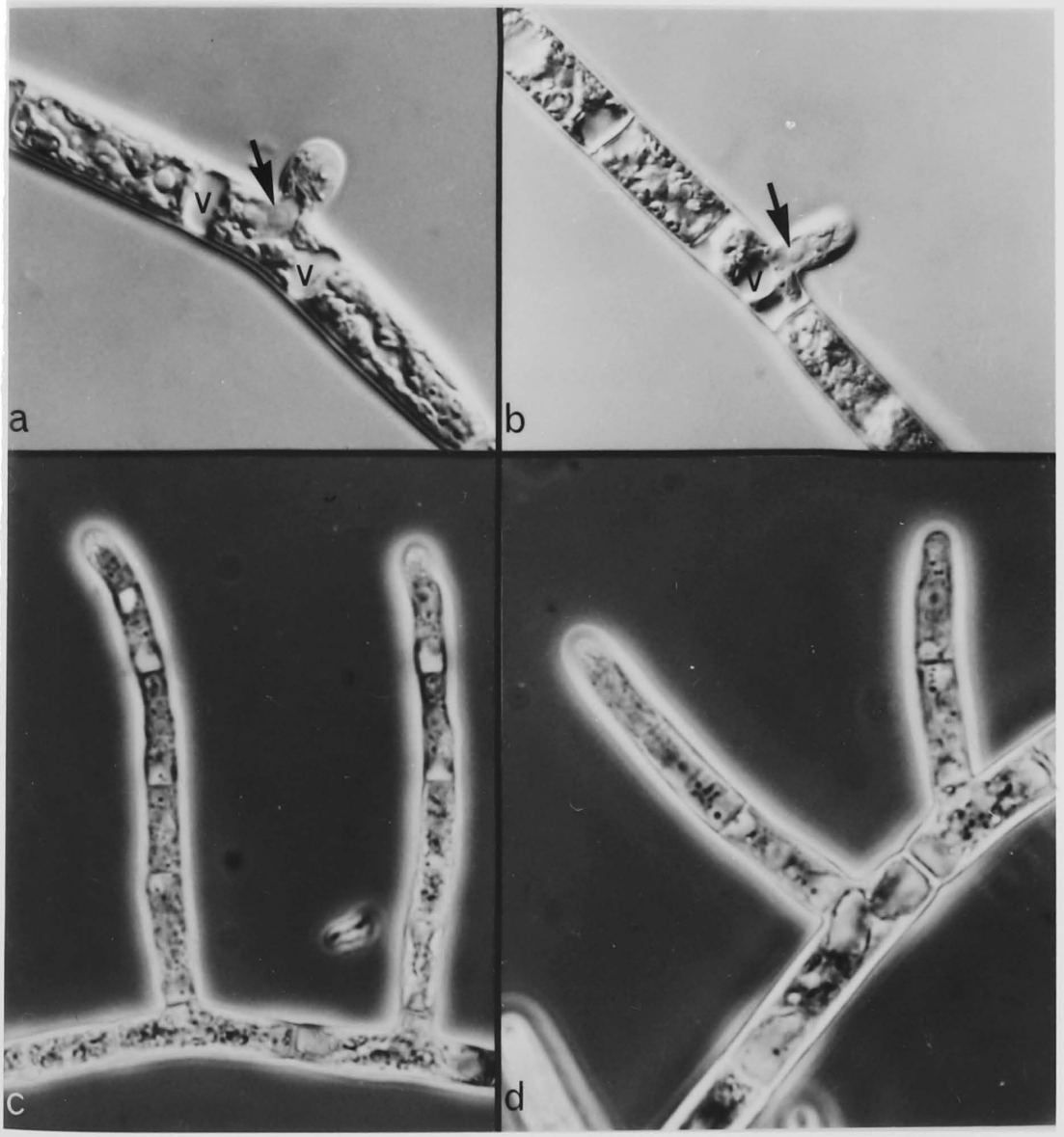
Fig. 3.6 Development of apolar branches at incipient plasmolysis in Stigeoclonium.

(a) Initiation of an apolar branch with vacuoles (v) still at either end of the cell. The central nucleus (arrow) has just started to move into the branch. Nomarski X850.

(b) A slightly later stage of apolar branch formation. The nucleus (arrow) has moved further into the branch and a large vacuole (v) has formed in the centre of the cell. Nomarski X700.

(c) Apolar branches after 72 hours at incipient plasmolysis. Note the irregular curves in the cell wall. Phase contrast X500.

(d) Unusual branch formation at incipient plasmolysis. One branch formed towards the end of the cell is apolar, while the neighbouring cell has produced a weakly polar branch from its centre. Phase contrast X800.


v
v
a
v
b
c
d

from the centre of a cell while an apolar branch was occasionally formed at approximately right angles to the main filament but from one end of the cell (Fig. 3.6d).

5. The effect of plasmolysis during the cell cycle

Plasmolysis can be regarded as an external signal resulting in branching while the onset of light (L, in this case at 1700 hours) could be regarded as a signal regulating transverse division. The interaction between these signals results in the branch patterns shown in Fig. 3.7 Plasmolysis at 1500 hours, two hours before the onset of light (L-2 hrs), allows the cell to become committed to branch initiation before the time to divide is signalled by the light. Plasmolysis at 1700 hours (L) results in approximately equal numbers of cells responding to the two coincident signals of light and plasmolysis which results in some cells dividing before they branch while others branch without an immediate preceding division. At 1900 hours (L + 2 hrs) most cells have two daughter nuclei but have not yet completed cytokinesis. Plasmolysis at this stage results in each of the daughter nuclei producing branches soon after cytokinesis is finished. Plasmolysis at 2100 hours (L + 4 hrs) and examination 24 hours later reveals a one-cell spacing of the branches, since there has been time for the completion of another division after plasmolysis. Cell death was common for plasmolytic treatment at 1700 hours (L), 1900 hours (L + 2 hrs) and 2100 (L + 4 hrs) hours.

Fig. 3.7 The Effect of plasmolysis during the cell cycle on branch patterns observed 24 hrs. after plasmolysis

(200 interbranch counts at each time)

Treatment Times:

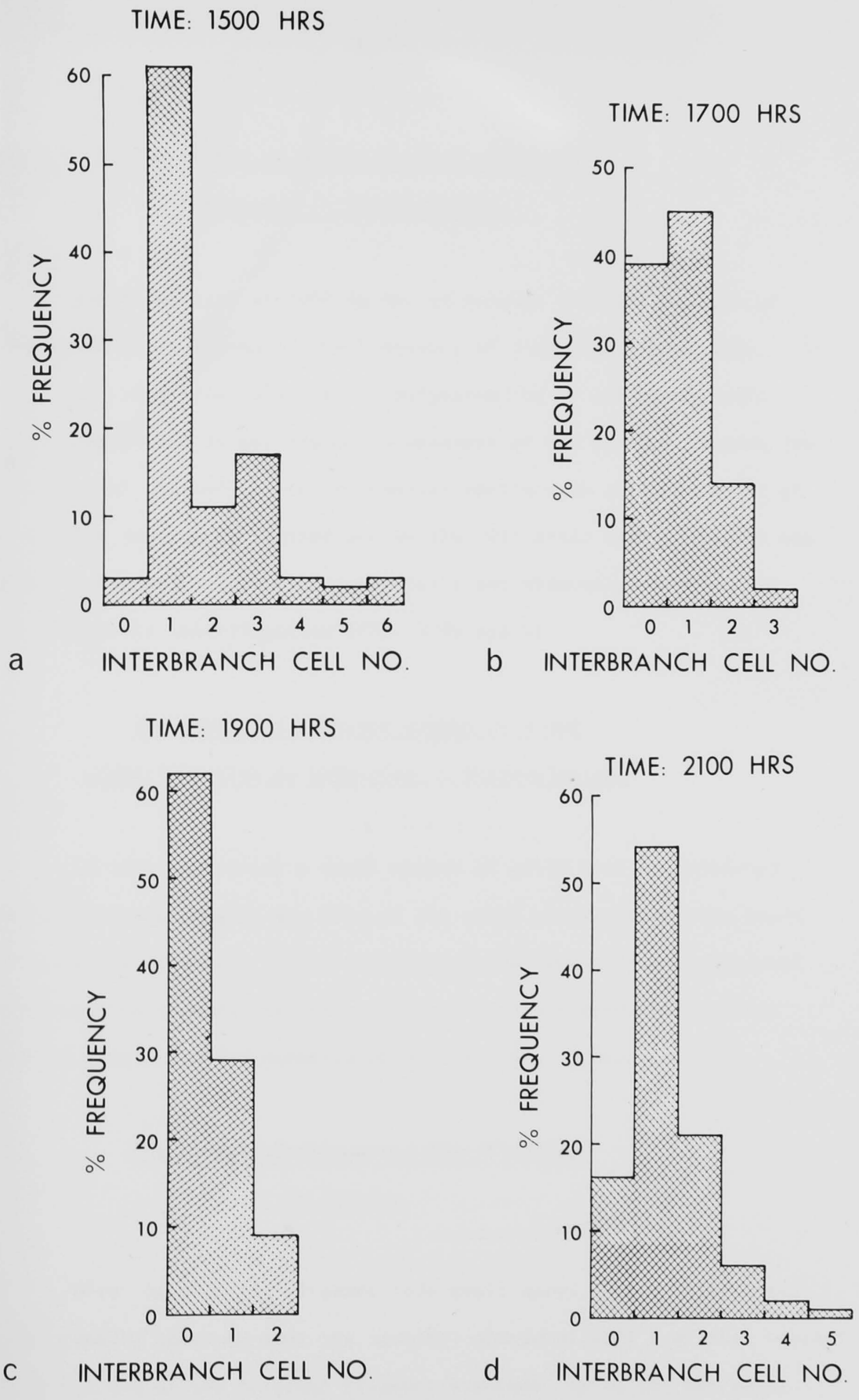
1500 hrs. - 2 hrs. before the onset of light.

1700 hrs. - onset of light.

1900 hrs. - 2 hrs. after the onset of light.

2100 hrs. - 4 hrs. after the onset of light.

Branch patterns were recorded exactly 24 hrs. after the respective time of plasmolysis.


TIME: 1500 HRS
% FREQUENCY
60
50
40
30
20
10
0
0 1 2 3 4 5 6
a
INTERBRANCH CELL NO.
TIME: 1700 HRS
% FREQUENCY
50
40
30
20
10
0
0 1 2 3
b
INTERBRANCH CELL NO.
TIME: 1900 HRS
% FREQUENCY
60
50
40
30
20
10
0
0 1 2
c
INTERBRANCH CELL NO.
TIME: 2100 HRS
% FREQUENCY
60
50
40
30
20
10
0
0 1 2 3 4 5
d
INTERBRANCH CELL NO.

6. The effect of centrifugation on branch patterns in Stigeoclonium.

Centrifugation at 1000 Xg for 30 minutes towards the apex of the plant clearly increased the frequency of branching (Table 3.3). It is presumed that this is due to a reorganisation of the cytoplasmic contents involving in particular, a movement of the nucleus towards the apical end of the cells. Using Nomarski optics such a displacement of the nucleus towards the apical end of the cell after centrifugation was commonly observed. Some bulging of cells and abnormal branching was also induced by centrifugation (Fig. 3.8a and b).

7. The effect of combined plasmolysis and centrifugation on branching in Stigeoclonium.

In this experiment a small number of cells that had produced branches growing towards the base of the plant after plasmolysis could be induced to produce a second branch from the same cell by subsequent centrifugation towards the apex. The two branches produced by these combined treatments had opposite polarities (Fig. 3.8c).

8. The effect of disrupting the filament by cutting

After cutting the filament into small parts with a razor blade it was readily observed that the terminal acropetal cell i.e. that nearest the apical end of the original filament branches, while the basipetal terminal cell produces rhizoids (Fig. 3.9). This results in regeneration

TABLE 3.3 THE EFFECT OF CENTRIFUGATION ON BRANCH FREQUENCY

| CENTRIFUGATION (30 minutes at 1000 X g) | Branch frequency (200 cells) |
|---|---|
| centrifuged | 47% |
| control | 21% |

Fig. 3.8 Centrifugation of Cells

(a) Bulging of cells after centrifugation.
Phase contrast X350.

(b) Some abnormal branching after centrifugation.
Phase contrast X350.

(c) Two branches produced by first plasmolysing a cell and then centrifuging the cell.
Nomarski X1,200.

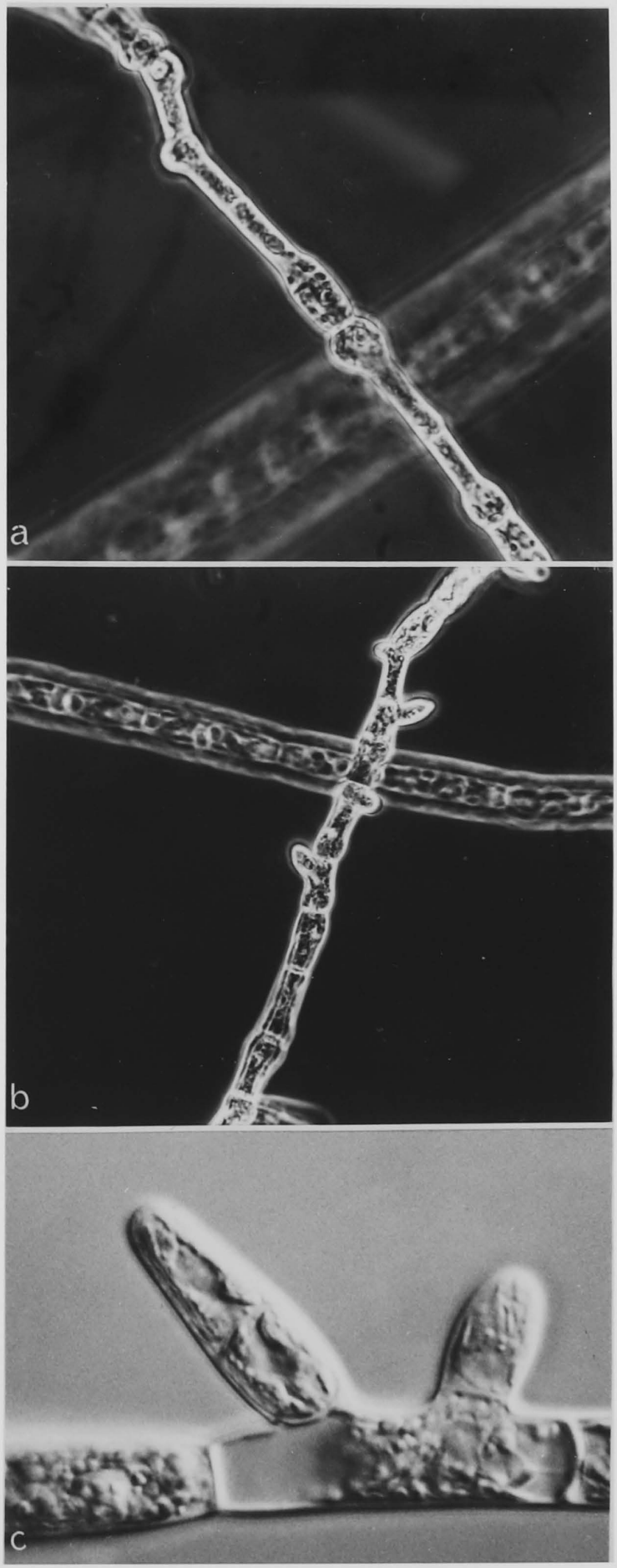

a
b
c

Fig.3.9 Surgical Experiments

(a) Electron micrograph of the cut end of a cell. Note the occlusion and deformation of the cell wall at the cut end (arrow). X12,000.

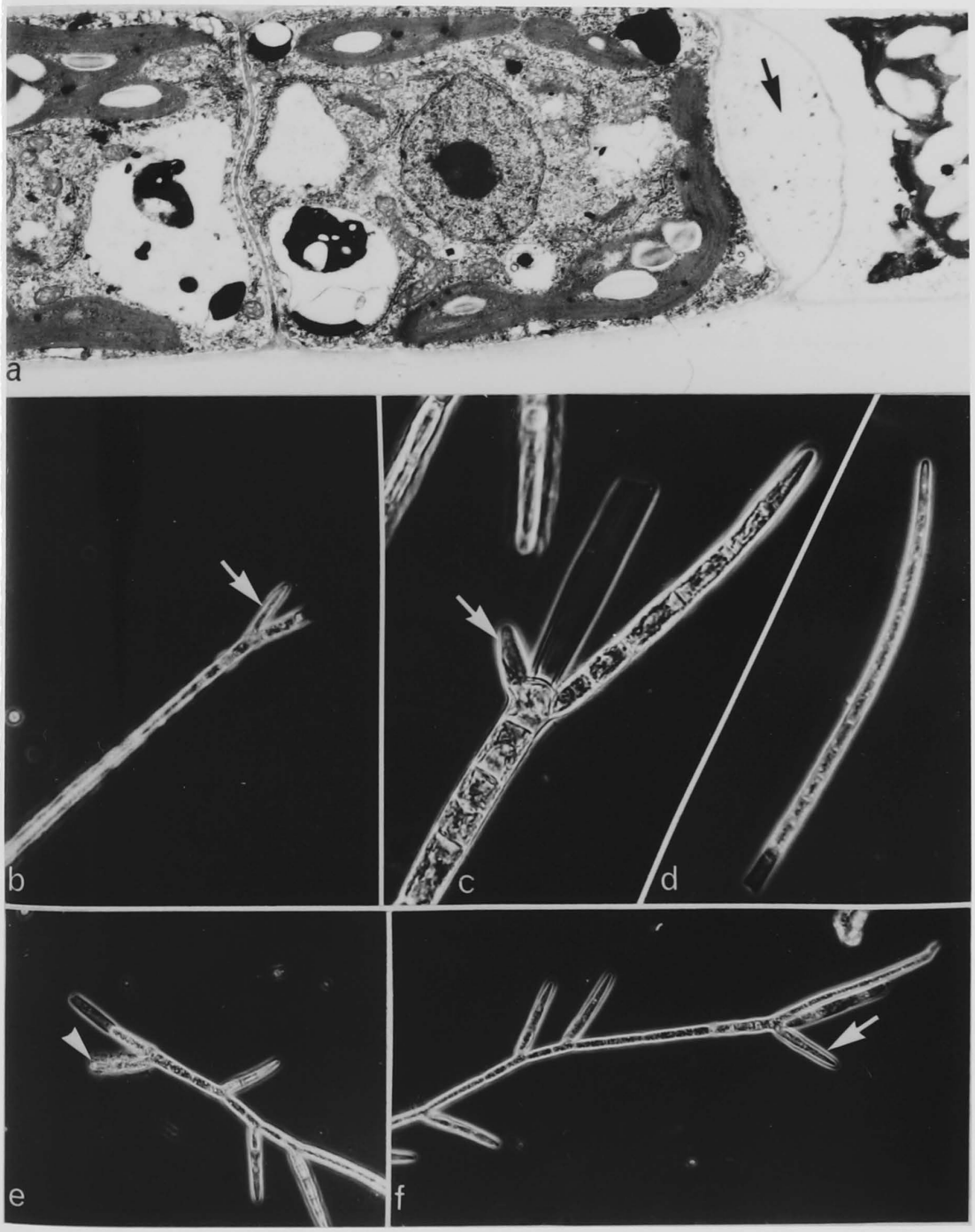
(b) Formation of a new apex (arrow) from the apical end of a cut fragment. Phase contrast X200.

(c) Formation of a second branch (arrow) from a fragment cut adjacent to an existing branch. Phase contrast X600.

(d) A severed apical fragment showing no branch or rhizoid formation. Phase contrast X250.

(e) and (f)

The basal (e) and apical (f) end of a single fragment. The basal end (e) shows rhizoid formation (arrowhead) while the apical end (f) shows branch formation (arrow). Phase contrast X200.


a
b
c
d
e
f

of a new individual in which the apico-basal polarity of the original plant is preserved in the form of an apical region and a rhizoidal holdfast. When a fragment is cut immediately adjacent to an existing branch a second branch is induced (Fig. 3.9c). However a severed apical fragment irrespective of its provenance, whether from the main axial filament or one of its branches, never formed a branch and often depending on the number of cells in the severed fragment, no rhizoid developed (3.9d). Rhizoid regeneration was more frequent from longer apical fragments and was only rarely observed from fragments containing less than 4 cells (Table 3.4. ). Under the electron microscope an occlusion and deformation of the cell wall was observed at the cut end (Fig. 3.9a).

9. Studies of branching in Stigeoclonium using a laser microbeam.

The laser microbeam with its spot size of approximately 3 µm (Fig. 3.10a) was used to kill specific vegetative cells in the filament of Stigeoclonium (Fig. 3.10c). Each experiment was repeated ten times.

In the first set of experiments apical cells were lased. In every case, a new branch point formed from the living cell immediately adjacent to the lased apical cell. The new branch point grew out and displaced the dead, lased apical cell (Fig. 3.11).

In the second set of experiments individual cells were isolated by laser shots from cells on either side. In a majority of cases (6 out of 10) the isolated cell produced a branch from its apical end, sometimes after a single division (Fig. 3.12a and b).

TABLE 3.4 THE EFFECT OF THE NUMBER OF CELLS IN APICAL FRAGMENTS ON THE RE-GENERATION OF RHIZOIDS

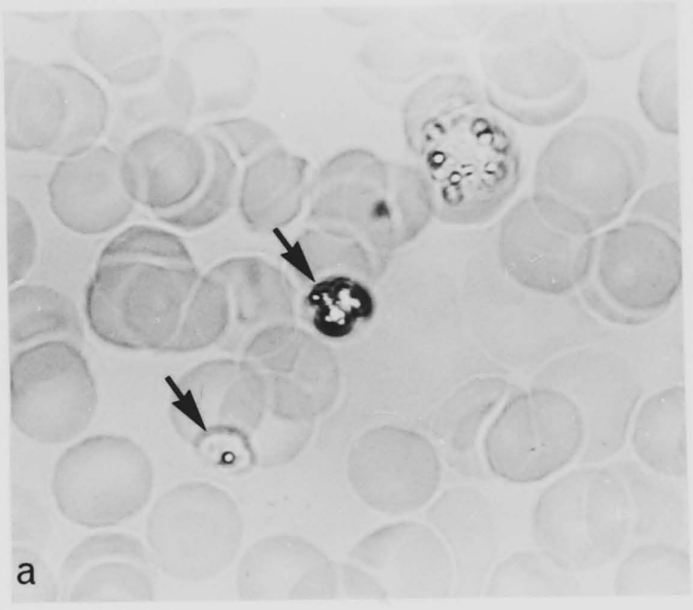
| No. of Cells in apical fragment (not including the apical cell) | Rhizoid Initiation (% from 100 fragments) |
|---|---|
| 2 | 3 |
| 3 | 12 |
| 4 | 32 |
| 5 | 72 |
| 6 | 78 |
| 7 | 85 |
| 8 | 92 |
| 9 | 87 |

Fig. 3.10 Laser Studies

(a) Human red blood smear used to establish the aiming mark and showing the size of the laser burn (arrows). The red blood cell is 7 µm wide. Phase contrast X1,400.

(b) Vegetative cell of Stigeoclonium. Phase contrast X1,200.

(c) Same cell after laser shot showing disorganization of cellular contents and deformation of end walls indicating loss of turgor (arrows). Phase contrast X1,200.


a

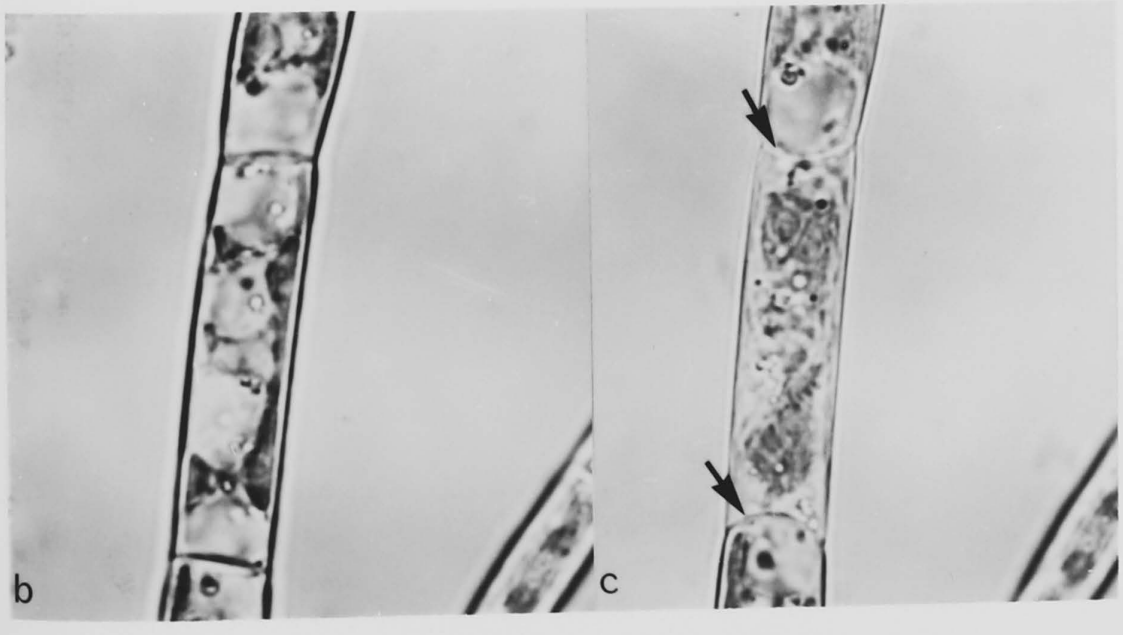

b
c

Fig. 3.11 Laser Studies

(a) Apical cell of Stigeoclonium. Phase contrast X800.

(b) Rupture and extrusion of apical cell contents after laser shot. Phase contrast X800.

(c) Initiation of a new apical cell (straight arrow) after killing the previous apex (curved arrow) with the laser. Phase contrast X350.

(d) Displacement of dead apical cell (curved arrow) by subsequent growth of the filament (straight arrow). Phase contrast X350.

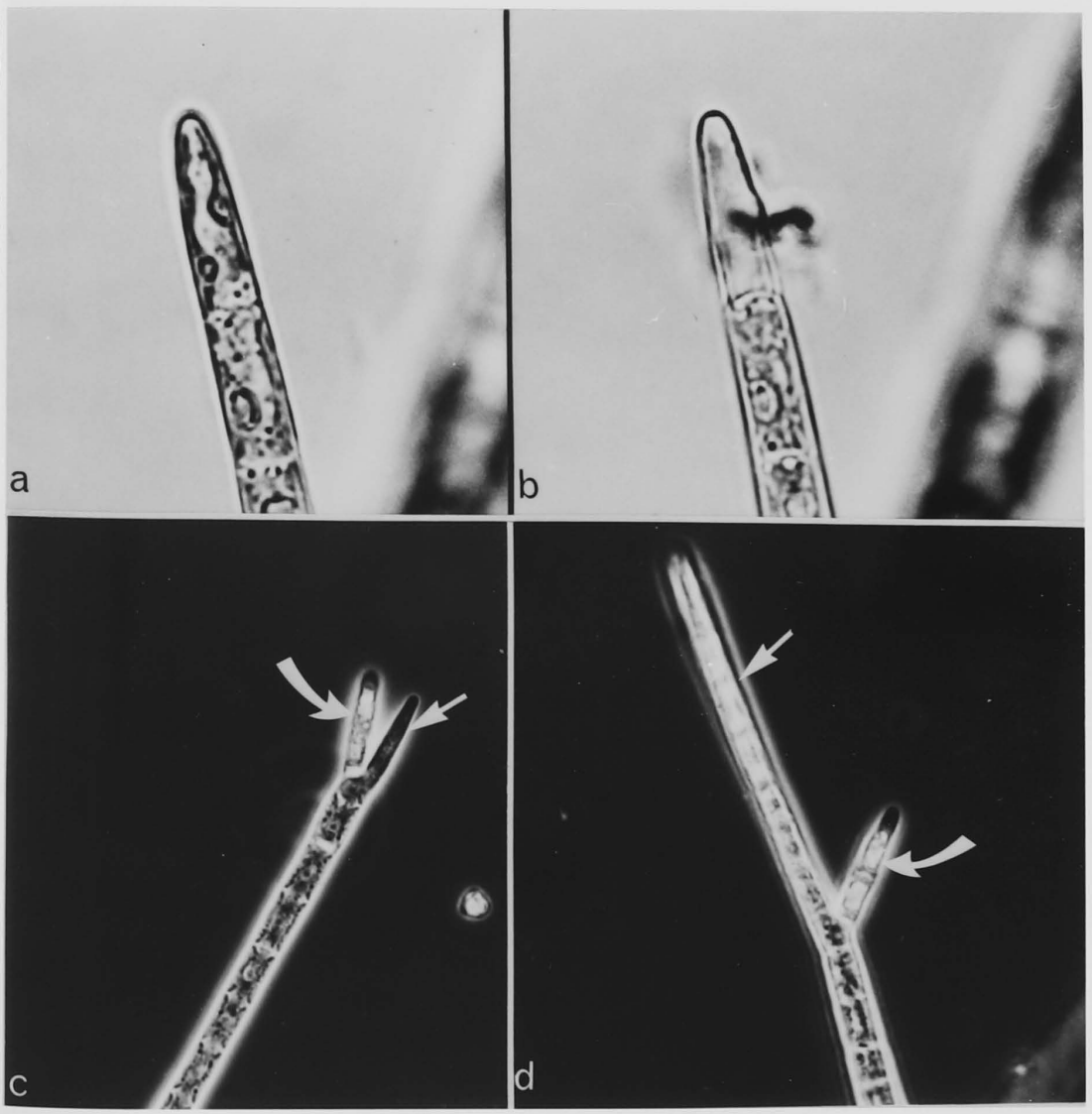

a
b
c
d

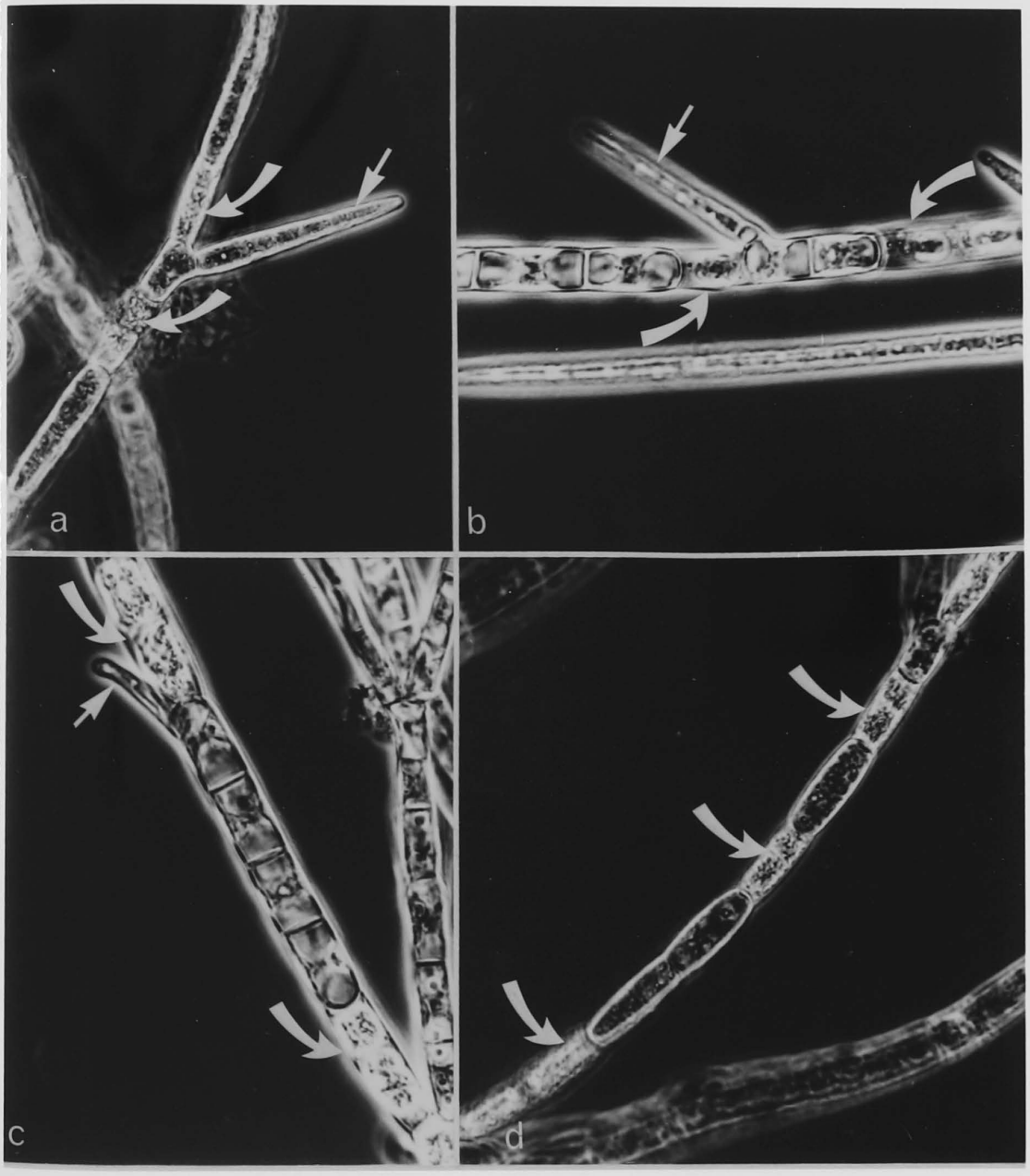
Fig. 3.12 Laser Studies

(a) Isolation of a single cell by lasing of the cells on either side (curved arrows) most commonly resulted in branch formation (straight arrow). Phase contrast X350.

(b) Sometimes the isolated cell underwent cell division and then produced a branch (straight arrow). Phase contrast X500.

(c) Isolation of a file of cells often resulted in the cell nearest the apex forming a branch (straight arrow). Phase contrast X600.

(d) Occasionally cells isolated by laser shots (curved arrows) did not form branches or rhizoids. Phase contrast X450.


a
b
c
d

The single isolated cell did not regenerate in 2 out of 10 cases (Fig. 3.12d).

The isolation of files of cells by two laser shots, one at either end, resulted in a branch being formed from the apical end of the cell file (Fig. 3.12c).

If a single cell was lased remote from any branches, the acropetally adjacent living cell always produced a rhizoid while the basipetal adjacent cell always produced a branch. Similar results could be obtained by using a needle to kill cells at random in the filament. Thus the killing of a single cell resulted in an expression of the underlying polarity of the filament.

In the third set of experiments cells were lased above an established branch point. Lasing of a cell immediately above a branch point resulted in another branch being formed, to produce a double branch point immediately below the lased cell and a rhizoid from the cell immediately above the lased cell (Fig. 3.13a and c). Lasing of a cell immediately above a double branch point did not result in any further branch initiation but a rhizoid was formed from the cell immediately above the lased cell (Fig. 3.13b). Lasing above a branch point so as to leave one cell between the lased cell and the branch point resulted in a similar polarity in the regeneration products, a branch being formed from the living cell above it (Fig. 3.14a and b). Single exceptions for these lasing shots above a branch involved the absence of rhizoidal development (Fig. 3.14d) or rhizoid formation from the apical end of

Fig. 3.13 Laser Studies

(a) and (c)

Lasing of a cell (curved arrow) adjacent to a branch point resulted in another branch (straight arrow) being initiated and a rhizoid (arrowhead) from the cell immediately above the lased cell. Both Phase contrast X500.

(b) Lasing of a cell (curved arrow) next to a double branch point resulted in a rhizoid (arrowhead) being formed from the cell immediately above the lased cell. Branch formation from the double branch point was not stimulated by lasing of the adjacent cells. Phase contrast X500.

(d) Occasionally no rhizoid formation was induced by lasing next to a branch point but branch formation (straight arrow) was still induced. Phase contrast X500.

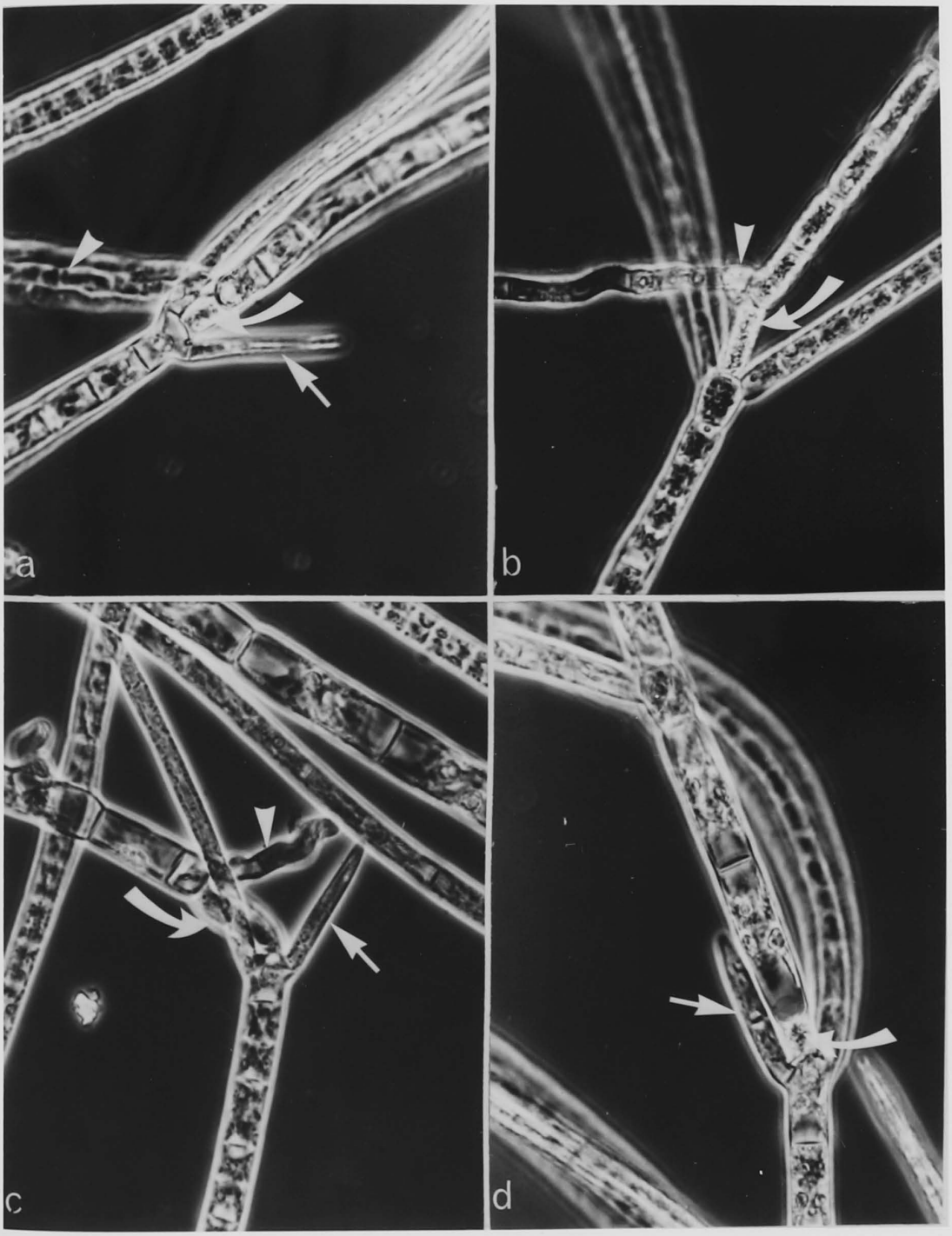

a
b
c
d

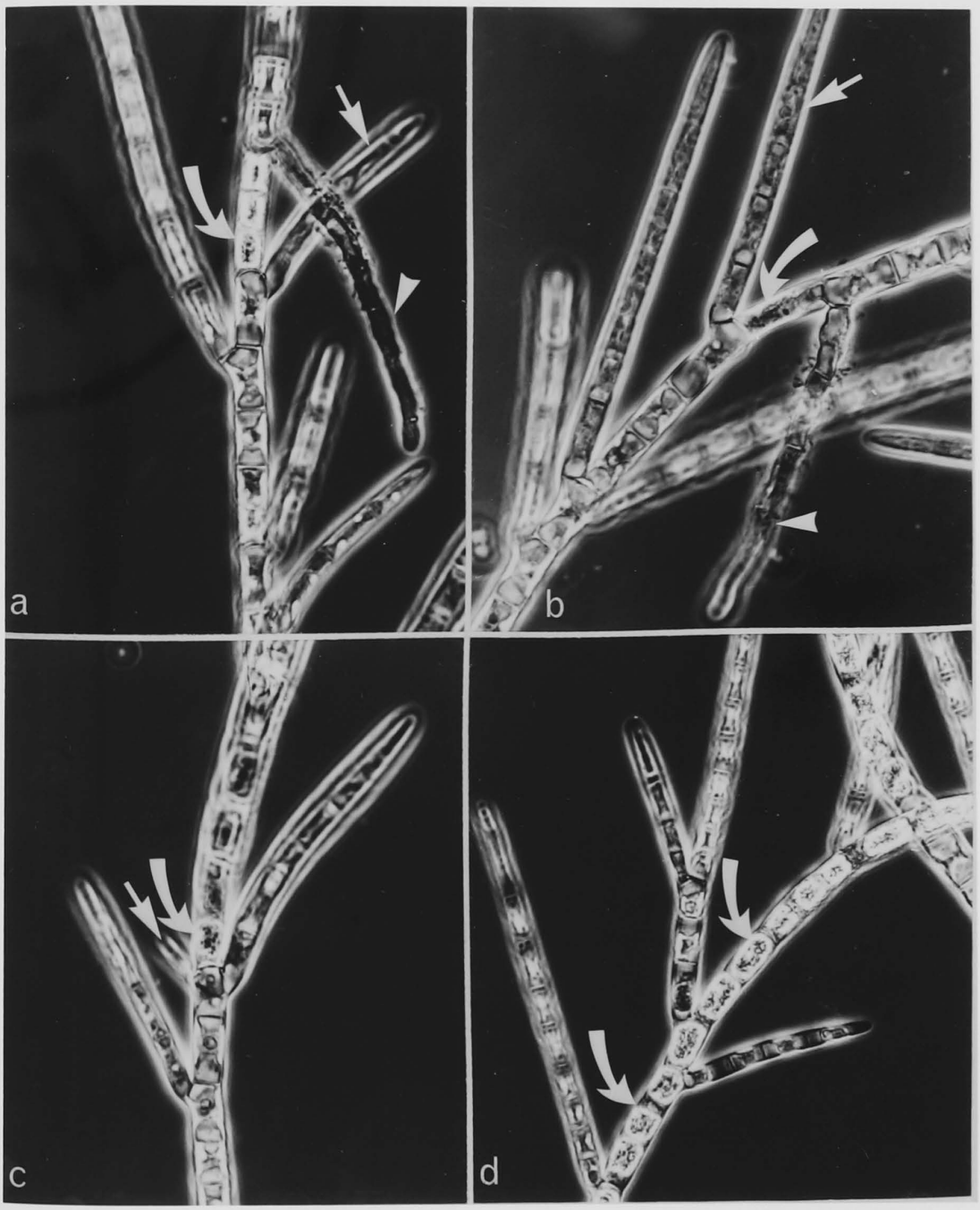
Fig. 3.14 Laser Studies

(a) Lasing cells (arrow) above a branch point so as to leave one living cell above the branch. This resulted in formation of a branch (straight arrow) from the living cell above the branch and a rhizoid (arrowhead) from the first living cell above the lased cell. Phase contrast X500.

(b) A situation similar to that in (a) but further removed from the branch gave the same results. Phase contrast X500.

(c) Lasing (curved arrow) next to an existing branch point resulted in the formation of a double branch point (arrowhead) but no rhizoid formation. Phase contrast X500.

(d) Spreading of heat from a number of laser shots (curved arrows) has led to death of many cells in a filament and no formation of new branches. Phase contrast X350.


a
b
c
d

cells (Fig. 3.16a and b). Some of these anomalous results may have been due to the spread of damage to the cell near the laser shot. The heat generated by the laser shots might spread into neighbouring cells, killing or injuring them and inhibiting regeneration (Fig. 3.14d).

In the fourth set of experiments cells were lased below an established branch point. This resulted in the formation of rhizoids from the acropetally adjacent cell (Fig. 3.15a and b). Lasing of a cell immediately adjacent to, but below a branch point often resulted in the formation of a rhizoid from the basal end of a cell that already bore a branch (Fig. 3.15c and d). Single exceptions for these lasing shots below a branch point involved the formation of the abnormal branch-like outgrowths shown in Fig. 3.16 c and d.

The lasing results highlight the underlying polarity of the system. If branching is considered to be regulated by inhibitors, the evidence would indicate that the branch inhibitor must be transmitted from the branch apex towards the base of the plant. Thus, the inhibitory field around each branch could be considered to have a strong vectorial component.

Fig. 3.15 Laser Studies

(a) and (b) Lasing of cells below a mature branch point (curved arrows) resulted in the formation of rhizoids (arrowhead) from the adjacent cell located towards the apex.
(a) Phase contrast X450,
(b) Phase contrast X250.

(c) and (d) Lasing of a cell (curved arrow) immediately adjacent to, but below a mature branch point often resulted in the formation of a rhizoid (arrowhead) from the basal end of a cell that already bore a branch (straight arrow).
(c) Phase contrast X600,
(d) Phase contrast X600.

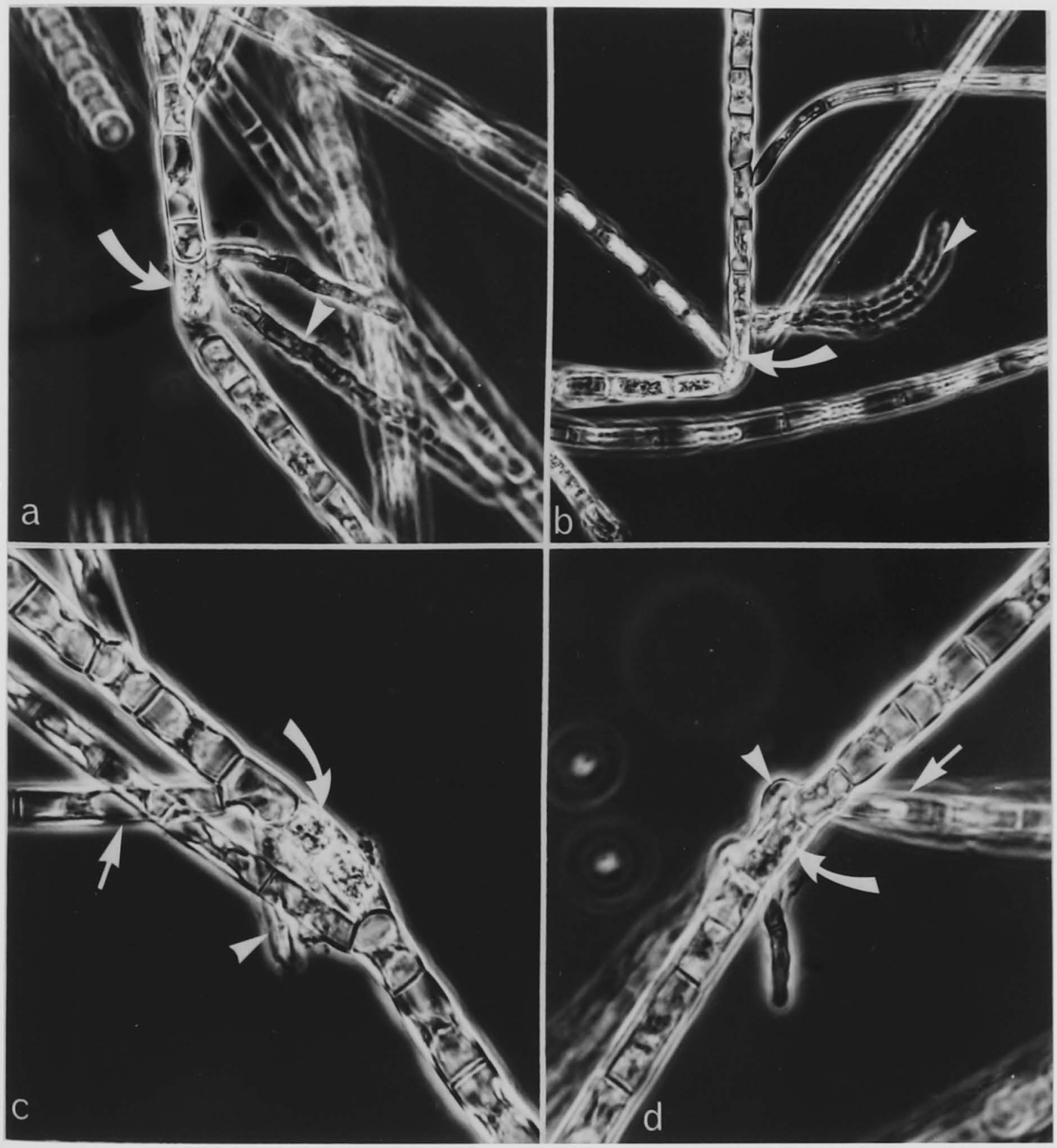

a
b
c
d

Fig. 3.16 Laser Studies - Atypical results.

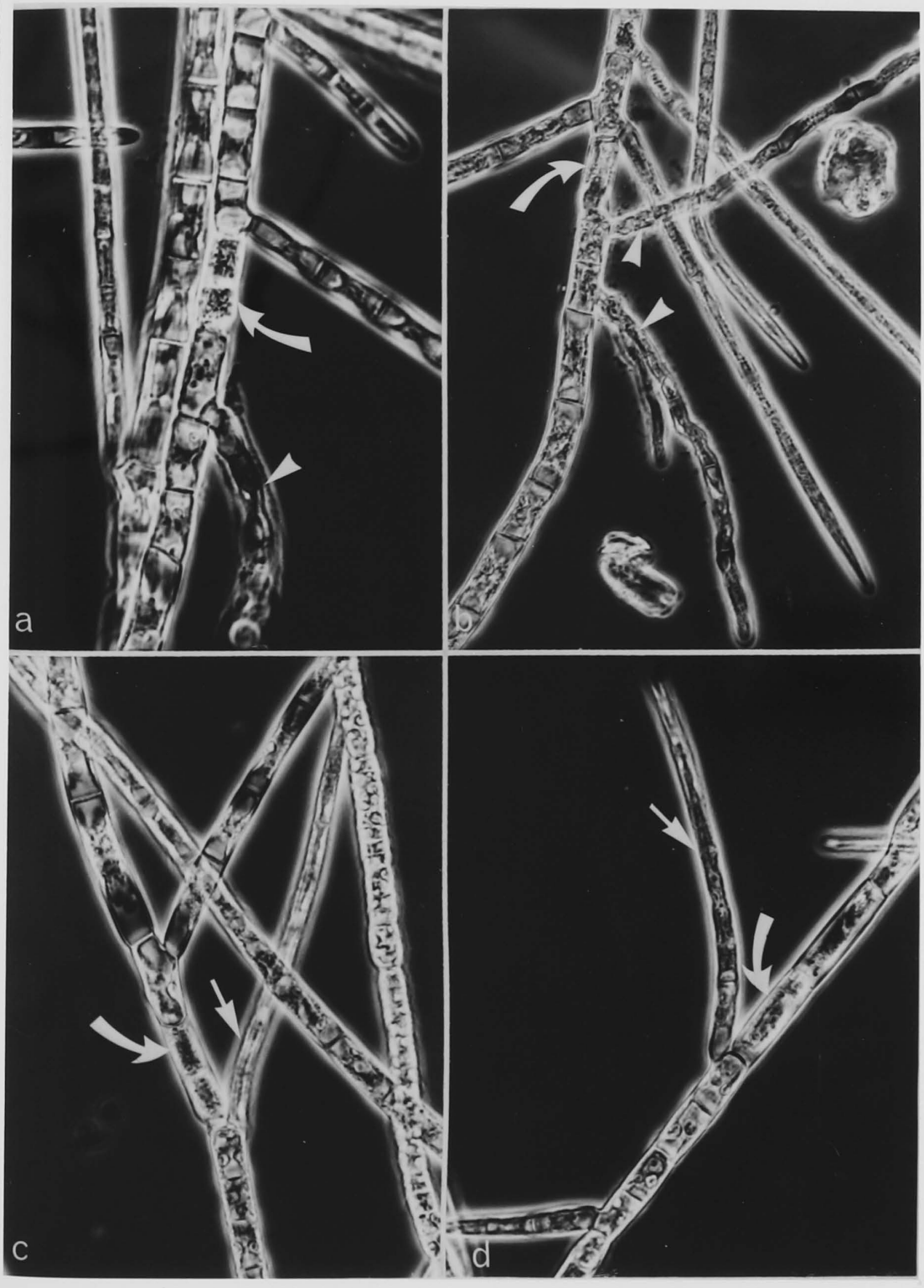
The apex of the plant is towards the bottom of Figs. a, b and d, but towards the top of Fig.c.

(a) Lasing (curved arrow) above a branch point resulted in a rhizoidal outgrowth (arrowhead) from the apical end of a cell where the branch is normally formed. Phase contrast X500.

(b) Lasing (curved arrow) above a branch point led to rhizoidal outgrowths (arrowhead) from two cells immediately above. One in the normal basal position, the other at the apical end of the cell. Phase contrast X450.

(c) Lasing (curved arrow) immediately below a branch resulted in a branch-like but very thin outgrowth (straight arrow) from the first living cell below the laser shot. Phase contrast X450.

(d) Lasing of a cell below a branch point (curved arrow) has led to a branch-like outgrowth (straight arrow) from the basal end of a cell where a rhizoid would normally be expected. Phase contrast X450.


a
b
c
d

## 3.3.2 Results for Drug Treatments of Stigeoclonium.

### 1. Colchicine and Cytochalasin B

The results of these experiments showed the differential effect of these two drugs upon the polarity of branch initiation after plasmolysis (Table 3.5). Cultures treated with cytochalasin B produced abnormally-shaped stunted branches which were formed as bulges from the middle of the cell (Fig. 3.17a and b). Branches did not grow past the first cell stage. When cytochalasin B was washed out after 48 hours of treatment some normal branch development was observed from the stunted initials.

Cultures treated with colchicine produced stunted branches from the apical end of the cell (Fig. 3.17c and d). When colchicine was washed out after 48 hours of treatment branches resumed growth and some normal branches eventually developed. Both sets of controls produced branches of normal shape and polarity. Branch initiation was not inhibited by either colchicine or cytochalasin B. Nuclear movement to one end of the cell during the initiation of branches was prevented by cytochalasin B but not by colchicine. This suggests that microfilaments are an essential component in the nuclear migration during polar branch development as discussed critically in 3.4. Both drugs inhibit the development of branches beyond the first cell stage and this could be due to the inhibition of cell division.

TABLE 3.5 THE EFFECT OF COLCHICINE AND CYTOCHALASIN B ON BRANCH POLARITY

| | Branch Polarity (% frequency for 200 branches) | | |
|---|---|---|---|
| | apical | central (apolar) | basal |
| Colchicine | 82% | 12% | 6% |
| Cytochalasin B | 22% | 74% | 4% |
| Control | 89% | 2% | 9% |

Colch. : control, Apical : Remainder, 1:1

$\chi_1^2$ = 3.4 (not quite significant at the 5% level)

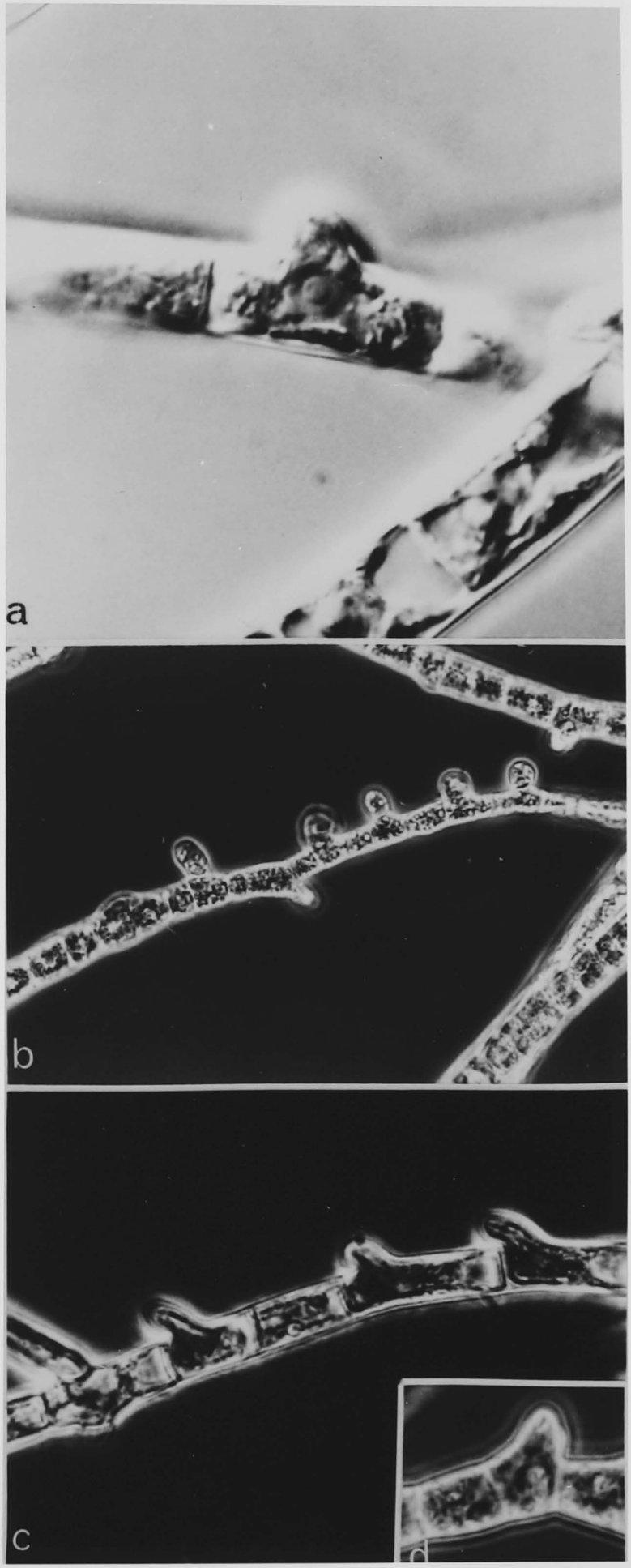
Fig. 3.17 Polarity of branching after treatment with cytochalasin B and colchicine.

(a) Initiation of an apolar branch during cytochalasin B treatment. Nomarski X1,000.

(b) Development of apolar but abnormally shaped branches during cytochalasin B treatment. Phase Contrast X250.

(c) Polar branches produced during colchicine treatment. Phase Contrast X500.

(d) Abnormally shaped polar branch produced during colchicine treatment. Phase Contrast X700.


a
b
c
d

## 2. Caffeine

The effect of caffeine treatment on plant cells is to prevent the fusion of vesicles involved in cell plate formation during cytokinesis after normal mitosis, thus producing bi-nucleate cells (Fig. 3.18a). Cells of Stigeoclonium treated with caffeine were subsequently plasmolysed. This technique produced a large number of apolar branches in adjacent cells (Fig. 3.18b). This would indicate that each nucleus responds to plasmolytic treatment and may produce a single branch. In most cases cytokinesis was completed before initiation of a branch from the previously bi-nucleate cell. However, in some cases a large bulge formed in the side of the cell, from which two branches subsequently developed (Fig. 3.18c). This would suggest that in such a case a bi-nucleate cell failed to complete its cytokinesis, that both nuclei passed into the large bulge at the side of the cell and that only subsequent to this was an individual branch developed from each nucleus. Caffeine-treated cells produced branches from all sides of the filament and occasionally two branches were formed from opposite sides of a single cell after plasmolysis (Fig. 3.18d).

## 3. 5- Aminouracil

Plasmolysis of cells whose cell cycle had been synchronised to the final portion of the S phase of interphase by prior treatment with 5-aminouracil (Diéz et al. 1976) resulted in a marked increase in branching frequency (Table 3.6) from long files of cells, with every cell producing a branch.

Fig. 3.18 Caffeine treated cells after plasmolysis.

(a) Binucleate cell (arrow) produced by 24 hour caffeine treatment. Nomarski X1,200.

(b) Caffeine treated cells after plasmolysis. Each adjacent cell has produced a branch, approximately at right angles to the main filament. Phase Contrast X850.

(c) Caffeine treated cells after plasmolysis. Initially a large bulge (arrow) was formed and subsequently two branches developed, but at different rates. Phase Contrast X1,100.

(d) Caffeine treated cells after plasmolysis. Branches are produced from all sides of the filament and occasionally two branches are formed from opposite sides of a single cell. Phase Contrast X500.

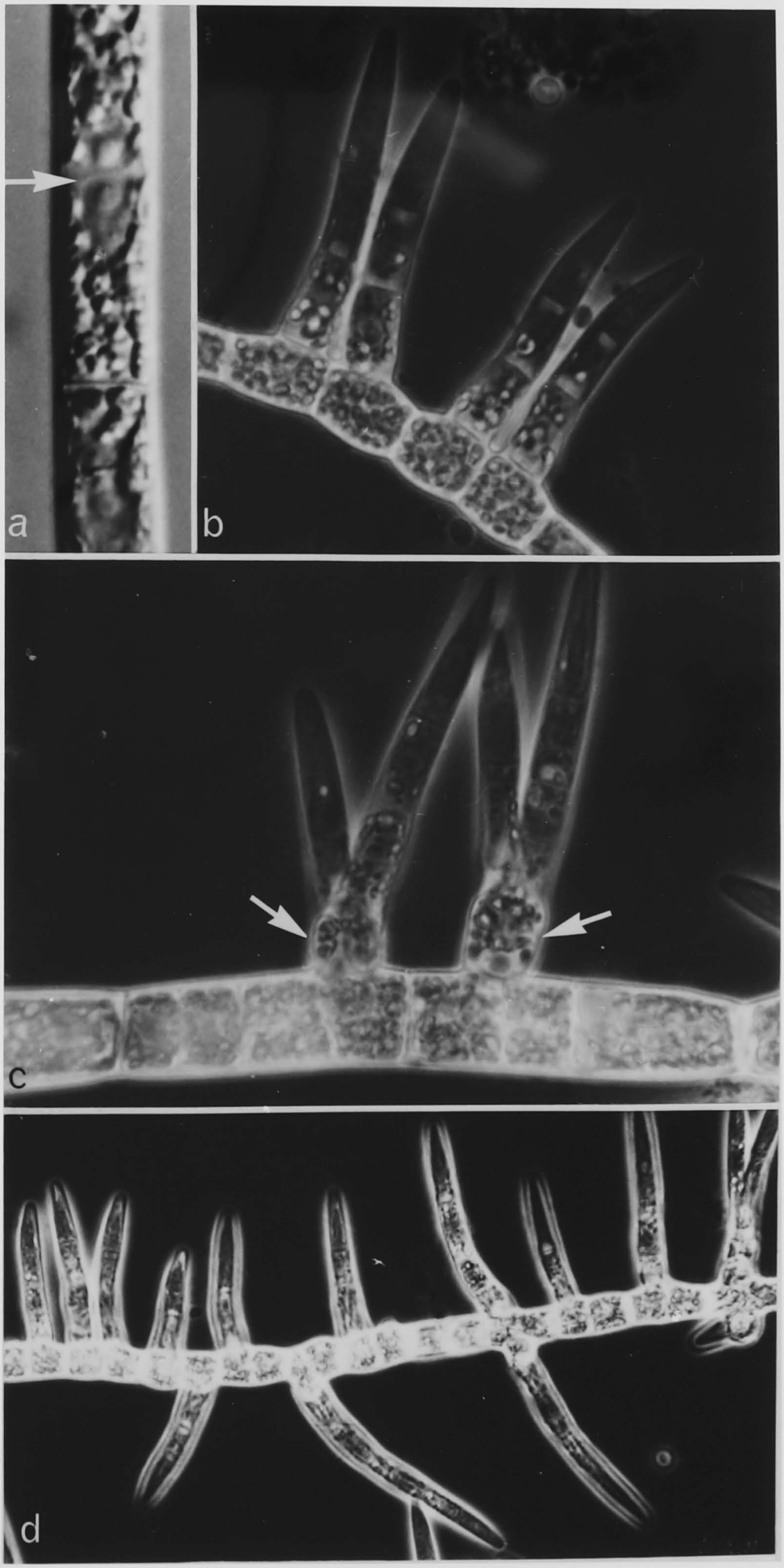

a
b
c
d

TABLE 3.6 THE EFFECT OF 5- AMINOURACIL ON BRANCHING FREQUENCY AFTER PLASMOLYSIS

| | Branching Frequency (200 cells) |
|---|---|
| 5- Aminouracil + plasmolysis | 74% |
| 5- Aminouracil | 18% |
| Plasmolysis | 39% |
| Control (culture medium) | 21% |

4. Actinomycin D and cycloheximide

Treatment with either actinomycin D or cycloheximide immediately after plasmolysis prevented the plasmolytic induction of branches (Table 3.7). However following incubation in cycloheximide the cells did not readily recover and interpretation of the results is difficult because of the considerable amount of cell death. Treatment with actinomycin D could readily be reversed by washing out the drug with fresh growth medium whereupon the normal branch polarity was seen to be preserved.

The results indicate that within the first three hours after plasmolysis the biochemical processes involved in branch initiation have already begun and in 17% of cells could not be suppressed by subsequent actinomycin D treatment. The longer the time that had elapsed between plasmolysis and treatment with actinomycin D, the higher the frequency of branch initiation. For at least 12 hours after plasmolysis branch initiation could be prevented entirely by cycloheximide.

The results from the actinomycin D experiments give some indication of the time course of cellular events during branch initiation. Placing cells in actinomycin D at a given time after plasmolysis permitted the determination of the degree of commitment to branch initiation which had taken place during that period. The results show clearly that between 3 and 6 hours after plasmolysis branch initiation is well under way. By plasmolysing and transferring cells immediately into actinomycin D branch initiation was prevented. If cells were plasmolysed and then kept in growth medium for three hours before

TABLE 3.7 THE EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON BRANCH INITIATION AFTER PLASMOLYSIS

| Beginning of Treatment after plasmolysis | % Frequency of Branch Initiation 24 hours after plasmolysis (200 cells counted) | | | |
|---|---|---|---|---|
| Hours | Act.D. | Recovery | Cyclohex. | Recovery |
| 0 | 0 | 42 | 0 | 0 |
| 3 | 17 | 46 | 0 | 0 |
| 6 | 21 | 45 | 0 | 0 |
| 9 | 32 | 43 | 0 | 16 |
| 12 | 36 | 45 | 18 | 26 |

| | | | |
|---|---|---|---|
| Control: | plasmolysed | 42 | 48 |
| | not plasmolysed (continuous drug treatment) | 6 | 0 |

transferring to actinomycin D some degree of branch initiation was observed. Thus it is within this first 3 hours after plasmolysis that the initial biochemical events leading to branch initiation must occur.

## 3.3.3 Results from Studies on intercellular communication in Stigeoclonium

### 1. Ultrastructure of plasmodesmata after plasmolysis

Twenty four hours after a standard plasmolysis treatment (see 3.2.1) an opaque staining of the cell wall was observed with the electron microscope (Fig. 3.19). In some cases few plasmodesmata could be seen traversing the cell wall (Fig. 3.19a). Cross walls in unplasmolysed young cells had only a very faintly staining deposit (Fig. 3.19c).

### 2. Callose deposition after plasmolysis

Callose deposits (identified by aniline blue fluorescence, see Discussion 3.4.2) were most commonly confined to the cross wall area following plasmolysis for 30 minutes in 0.6M mannitol in culture medium (Fig. 3.20a). Sometimes callose deposition was more widespread, and callose occurred in a large number of small areas throughout the cell (Fig. 3.20b) or as large callose plugs (Fig. 3.20c). Massive callose plugs were often associated with branch formation of reversed polarity (Fig. 3.20d) and cells that had been killed by plasmolysis.

### 3. The role of calcium in intercellular communication in Stigeoclonium.

#### (i) The interaction between calcium and callose

The previous section has shown that callose formation during

Fig. 3.19 Plasmodesmata in Stigeoclonium after plasmolysis and recovery

(a) After plasmolysis an opaque staining of the wall occurs (arrow) and few plasmodesmata can be clearly seen.
X33,000.

(b) A dark band (arrow) is also visible in this cross wall after plasmolysis but plasmodesmata seem to clearly traverse it.
X30,000.

(c) Cross wall from an unplasmolysed young cell has only a very faint deposit (arrow) in the cross wall. (t, microtubules).
X44,000.

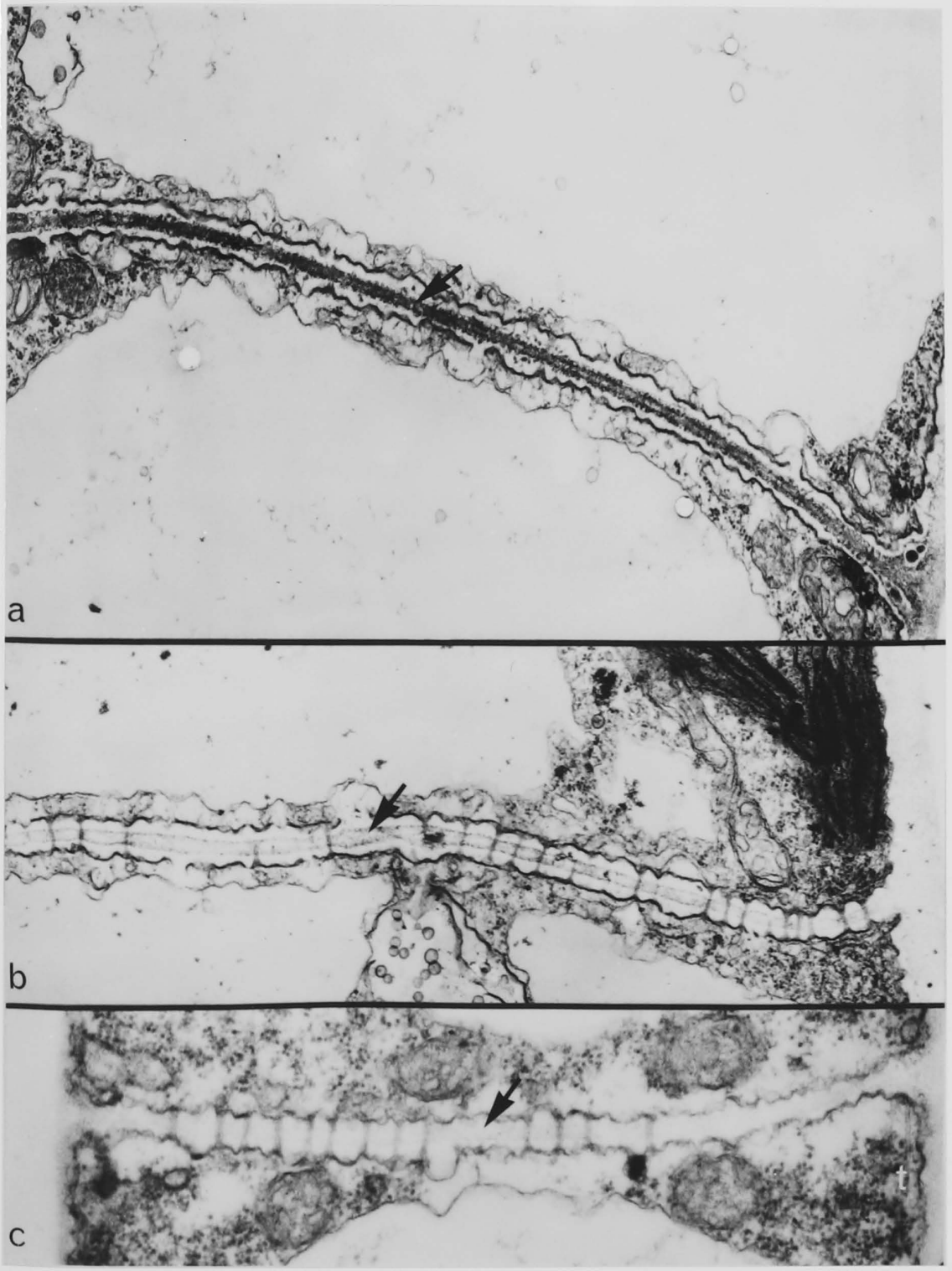

a
b
c
t

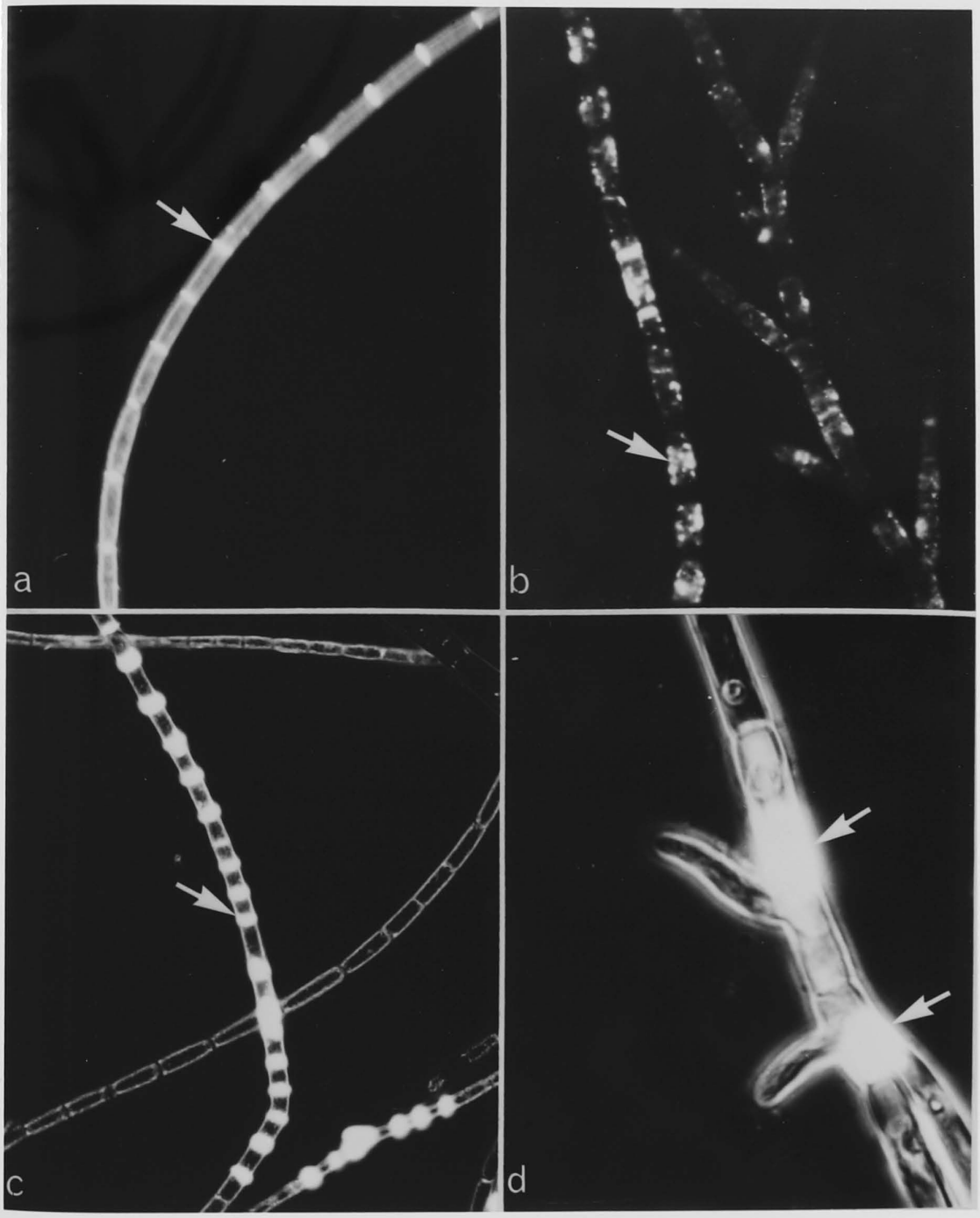
Fig. 3.20 Callose deposition after plasmolysis

(a) Callose deposits (arrow ) confined to the cross wall area after plasmolysis. Phase contrast and fluorescence microscopy X250.

(b) More widespread callose reaction after plasmolysis. Phase contrast and fluorescence microscopy X350.

(c) Large callose plugs after plasmolysis. Phase contrast and fluorescence microscopy X250.

(d) Large callose plugs and formation of branches with opposite polarity after plasmolysis. Phase contrast and fluorescence microscopy X500.


a
b
c
d

plasmolysis may be one way that the Stigeoclonium cell can block intercellular communication. In animal cells it has been established that calcium has a central role in preventing cell communication through gap junctions. Loewenstein (1968) has shown a build-up of calcium on either side of gap junctions during the breakdown of intercellular communication in animal cells. Experiments were set up to investigate the interaction between calcium and callose in the blockage of plant plasmodesmata.

Free calcium appears to be required for the formation of callose near the cross walls of Stigeoclonium as a result of plasmolysis. Callose will not form during plasmolysis if calcium is first chelated by EDTA and the cells are kept in a calcium-free medium (Table 3.8). However, immediately the cells are returned to a medium containing calcium, callose forms. The mechanism of the interaction between calcium and callose is not known. It could be that calcium is needed for some enzyme that produces a callose precursor or that calcium in some way gels the callose, as has been noted for alginates (Bardseth, 1965; Smidsrød and Haug, 1972).

(ii) Calcium ionophore studies

In many developmental processes an influx of calcium ions is an initiating signal, e.g. during fertilisation of the sea urchin egg a calcium influx occurs and the initial stages following fertilisation can be produced using a calcium ionophore (Steinhardt and Epel, 1974). In this experiment it was decided to study the effect of treatment with calcium ionophore A 23187 on the branching response

TABLE 3.8 THE EFFECT OF CALCIUM ON CALLOSE DEPOSITION DURING PLASMOLYSIS

| Treatment | % septa with aniline blue fluorescence staining after plasmolysis (200 counts) |
|---|---|
| EDTA treated in calcium free medium then plasmolysed. | 8 |
| Control in culture medium (no plasmolysis) | 7 |
| EDTA treated in calcium free medium, plasmolysed, then returned to culture medium containing calcium. | 68 |
| Control in culture medium after plasmolysis. | 72 |

in Stigeoclonium cultured in BBM containing $10^{-3}$ M $Ca^{2+}$. A 48 hour treatment of filaments with the ionophore did not result in a marked increase in branching frequency during treatment but upon release from the ionophore treatment a marked increase in branching frequency was recorded with many branches of basal polarity. A 48 hour treatment with the ionophore also led to an increased number of stunted branches with basal polarity (Table 3.9).

No marked callose deposition was noted in cultures treated with the calcium ionophore.

(iii) EDAX Studies

EDAX counts from the cross wall area where callose accumulates during plasmolysis (Fig. 3.21b) show a distinctive peak for calcium (Figs. 3.21c and d) which was not present in similar areas near the cross walls of unplasmolysed cells (Fig. 3.21a). The data suggests that calcium may accumulate in the cross wall area during plasmolysis.

(iv) Growth Studies in calcium-free Bold's Basal medium

Growth of Stigeoclonium in calcium-free BBM resulted in an increase in branching frequency, irregularity of branch polarity, bending of filaments, a reduction in volume of the cytoplasmic contents, particularly the chloroplasts and increased vacuolation (Fig. 3.22).

TABLE 3.9 THE EFFECT OF TREATMENT WITH THE CALCIUM IONOPHORE A 23187 ON BRANCH INITIATION

| | % Branching Frequency (200 cells counted) | Branch Polarity (200 branches recorded) | | |
|---|---|---|---|---|
| | | apical | Central apolar | basal |
| Ionophore treated (48 hours) | 27 | 57 | 11 | 32 |
| Release from ionophore | 53 | 48 | 13 | 39 |
| Control | 24 | 92 | 1 | 7 |

Fig. 3.21 EDAX Studies

(a) EDAX data for an area near the cross wall of an unplasmolysed Stigeoclonium cell fixed in glutaraldehyde and post fixed in osmium. (Ca, calcium; Os, osmium; Cl, chlorine; Mg, magnesium).

(b) Section of a cross wall area of a plasmolysed Stigeoclonium cell. The area analysed is arrowed. X17,000.

(c) and (d)

EDAX data from the cross wall areas of plasmolysed Stigeoclonium cells. Note the peaks for calcium.

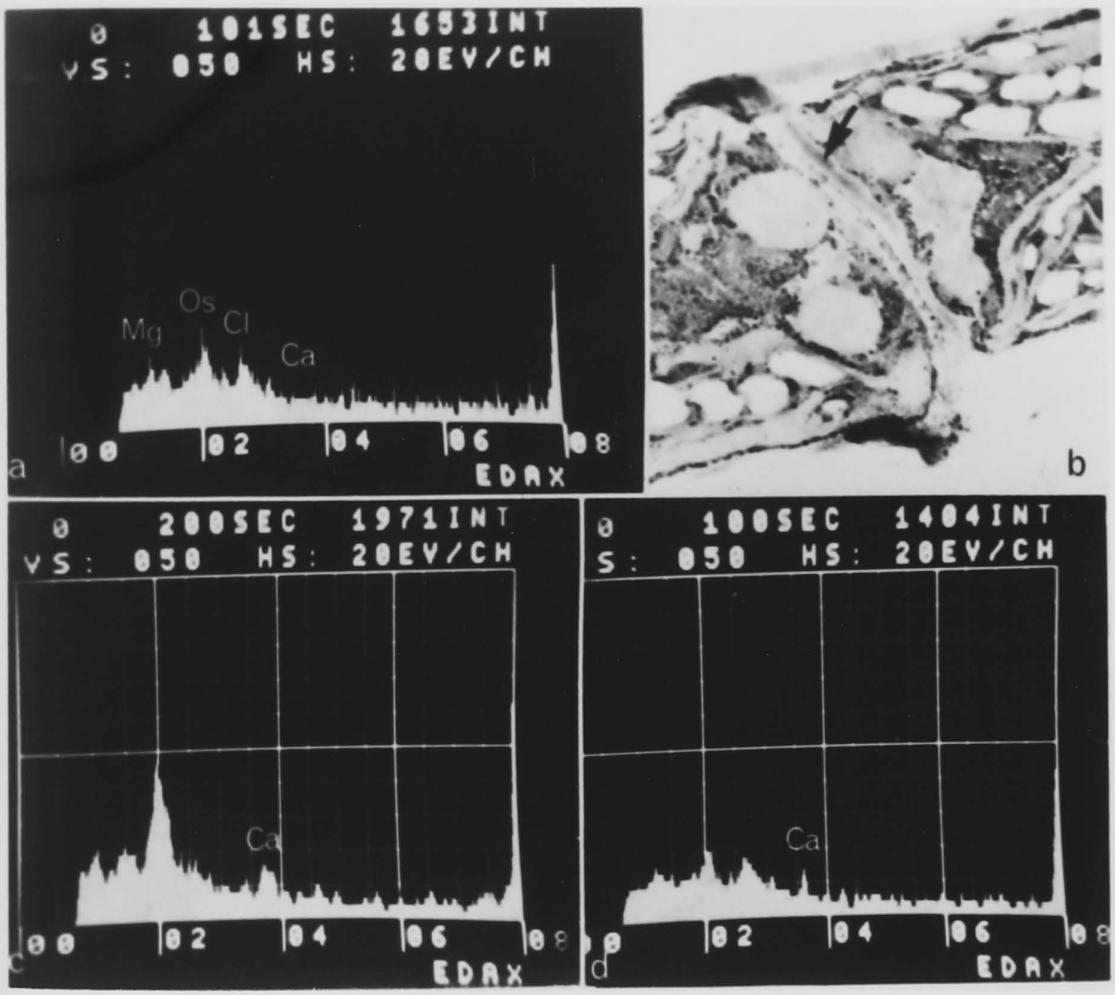
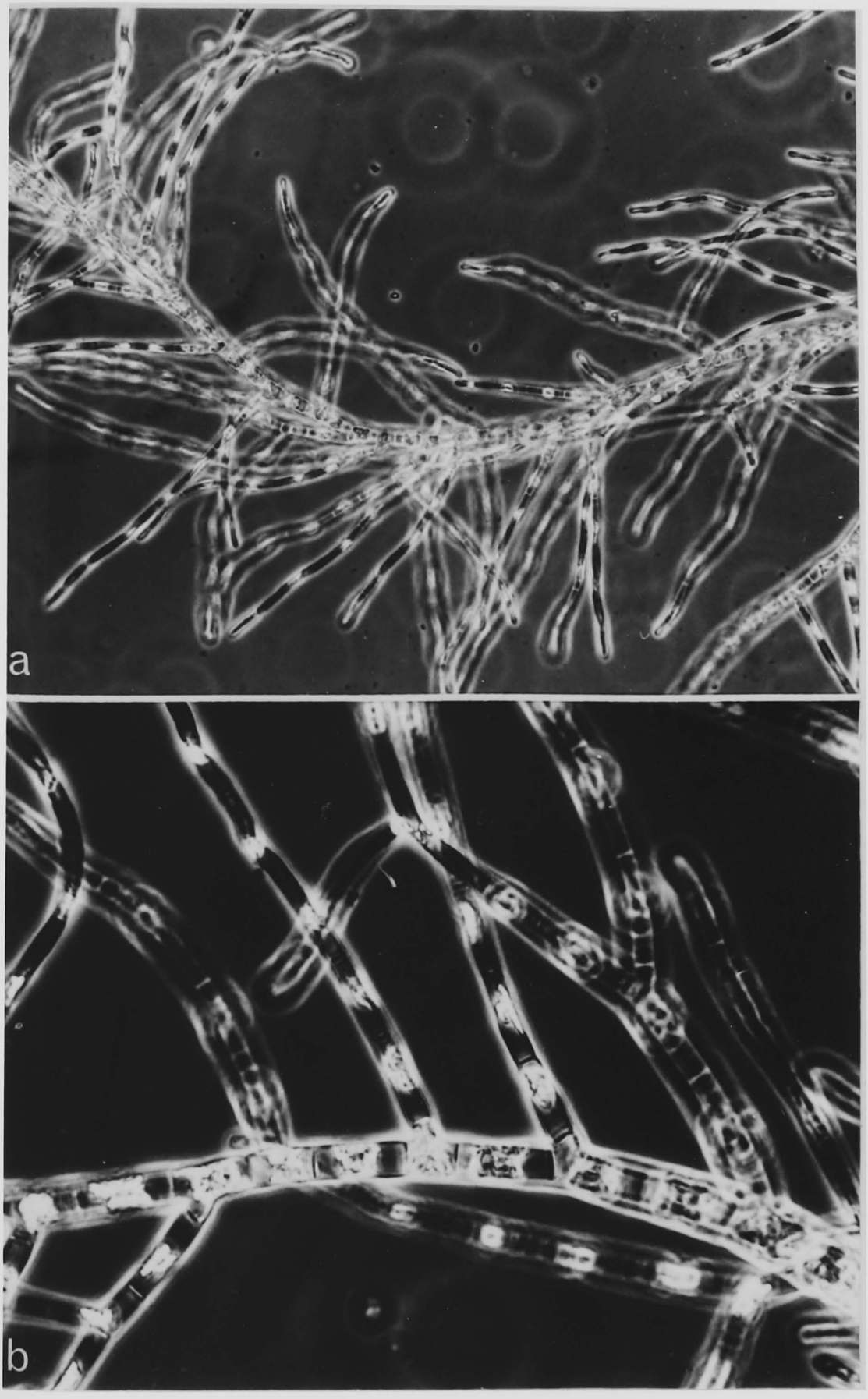

0 101SEC 1653INT
VS: 050 HS: 20EV/CH
Mg
Os
Cl
Ca
00
02
04
06
08
EDAX
a
b
0 200SEC 1971INT
VS: 050 HS: 20EV/CH
Ca
00
02
04
06
08
EDAX
c
0 100SEC 1404INT
S: 050 HS: 20EV/CH
Ca
02
04
06
EDAX
d

Fig. 3.22 Growth form of Stigeoclonium in calcium-free Bold's Basal Medium

(a) Branch development shows irregularity of polarity and bending of filaments. Phase contrast X200.

(b) Cells have reduced cytoplasmic contents, particularly the chloroplasts. Phase contrast X450.


a
b

4. Fluorescein diacetate transport studies

Although persistent attempts were made to monitor the symplastic transport of FDA through the filaments of Stigeoclonium no success was forthcoming because the integrity of the Valap seal around the wet filaments could never be maintained for long enough periods. These transport studies require either a more effective sealing agent or the intracellular injection of FDA.

5. Intercellular electrical coupling

Resting potentials of the order of -80mV were recorded for Stigeoclonium cells. However, despite persistent attempts, monitoring of these resting potentials could not be maintained during attempts to inject current with a second electrode in an immediately adjacent cell.

These problems were due to the small size of Stigeoclonium cells and the difficulty in securing the cells to allow insertion of the electrodes. Even after embedding in agar, cells were difficult to impale with electrodes because of movement of the filaments. The success of such studies on Stigeoclonium may largely depend upon a reliable technique for securing and stabilising the filaments to allow electrode insertion into adjacent cells.

## 3.4 DISCUSSION OF RESULTS

### 3.4.1 Physical Treatments

#### Effect of temporary plasmolysis on branching patterns in Stigeoclonium

Light microscopic observations of adjoining cells showed that plasmolysis produced a similar retraction of both protoplasts from the adjoining septa within a majority of the cells (Fig. 3.1a). However in some cases one protoplast retracted completely from the adjoining septum while the other was only partially retracted (Fig 3.1b). No polarity in the time-course of retraction was observed for individual cells. In the majority of individual cells protoplasts appeared to separate from both end walls simultaneously, in contrast to the polarity of plasmolysis observed in ferns.

In most cases the cytoplasm of individual fern prothallial cells retracts initially from the apical wall (Igura, 1955; Nakazawa and Ootaki 1961, 1962). In ferns, basal cells of both protonema and prothalli plasmolyse first followed gradually by cells closer to the apical regions (Nagai, 1915; Reuter, 1953; Igura, 1955).

By contrast Ito (1962) in his studies on Pteris prothalli demonstrated an inverse relationship between the age of a cell and the time it required after isolation to form the first cell of a new protonema. His studies were made on cells isolated by pricking and

killing the surrounding cells with a fine glass needle.

The difference in plasmolytic behaviour of Stigeoclonium and the fern prothallus may be due to the fact that Stigeoclonium grows by intercalary cell division while the fern prothallus grows by apical growth. Thus gradients in cell age are established during the development of the fern prothallus and these may underlie the gradients in plasmolysis.

The effect of plasmolysis on regeneration in plants has been attributed to the physiological isolation of individual cells from the correlative inhibition of neighbouring cells (see Carr, 1976). Temporary plasmolysis of fern prothalli leads to regeneration of filamentous outgrowths from individual cells (Nagai, 1914; Nakazawa, 1963). The correlative inhibition is presumed to be transmitted via the plasmodesmata which are said to be broken or blocked during plasmolysis. I presume that this is also the case in Stigeoclonium. Although there is no doubt that severe plasmolysis for prolonged periods will break plasmodesmata, it is unlikely that this is necessary for the physiological isolation of cells. Plasmolysis of the root meristem of Triticum and the coleoptile of Avena in 0.5M mannitol for one hour caused a shrinkage of the protoplast away from the cell wall but the plasmodesmata remained intact in the majority of cases (Burgess, 1971). Plasmolysis of the root meristem of Dryopteris in 0.8M mannitol for one hour resulted in most but not all plasmodesmatal connections being broken by the contraction of the protoplast. Plasmolysis of the gametophyte of the fern Asplenium nidus with 0.8M mannitol for 1-2 hours did not result in all plasmodesmata being broken on both sides of the cell walls

(Burgess, cited as pers. comm. in Graham and Wareing, 1977). In oat coleoptiles severe plasmolysis "either breaks plasmodesmatal connections or leaves the protoplasts still connected by strands of cytoplasm (Hechtian strands). Plasmolysis also induces the formation of callose around the plasmodesmata. The callose remains for several hours..." (Drake et al. 1978). Thus although plasmolysis can break plasmodesmatal connections it is likely that physiological isolation of cells leading to regeneration may be achieved more subtly, as discussed below. Indeed the results with Stigeoclonium suggest that very short osmotic shock treatments are sufficient to cause branching.

Rather than breaking plasmodesmata, osmotic shock may block them by the induction of callose deposition. It has been established that plasmolysis induces callose formation (Currier and Strugger, 1956; Eschrich, 1957; Drake et al. 1978). Carde (1974) has shown plasmodesmata to be surrounded by a callose sheath while Knox and Heslop-Harrison (1970) found that callose may temporarily isolate cells of the angiosperm meiotic tetrad by acting as a permeability barrier to large molecules. Thus a callose sleeve around plasmodesmata might act as a sensitive and effective "valve" in intercellular communication. Callose deposition is discussed more fully in Section 3.4.3.

The development of branches in Stigeoclonium after plasmolysis would require both enzymic softening of the existing cell walls and deposition of wall material for the new branch. There is evidence that plasmolysis can affect both these processes.

The plasmolytic induction of autolytic enzymes has been shown for yeast (Berliner and Reca, 1970; Berliner, 1971) and it is a possible mechanism for protoplast formation in the alga Cosmarium turpinii during treatment with 0.4M mannitol (Berliner and Wenc, 1976).

Evidence from some higher plants suggests that there is an accumulation of wall material by cells stressed by plasmolysis, which on recovery produces a 'growth burst'. Several workers (Ray, 1961; Green, 1968; Hsiao et al. 1970) have described a 'growth burst' when cells are returned from low to high turgor conditions. A similar "stored growth" effect has also been noticed for the reversible KCN inhibition of growth (Ray, 1961; Gillbank et al. 1972). Robinson and Cummins (1976) have studied the short-term effects of plasmolysis (30 minutes in 0.45M mannitol) and deplasmolysis on the Golgi secretion pathway in Pisum. They concluded that in plasmolysed cells the dictyosomes continue to function but incorporation of their products into the cell wall is inhibited so that the secreted material accumulates during plasmolysis, most probably between the protoplast and the cell wall. If, as seems most likely (Preston, 1974), cellulose synthesis occurs at the plasmalemma then the disruption of the plasmalemma-cell wall association by plasmolysis would be expected to affect cellulose synthesis.

Thus the "stored growth" effect of low turgor appears to reside in an inhibition of the secretory pathway at the sink i.e. the cell wall, while the "stored growth" effect of KCN treatment appears to be due to

an effect on the source i.e. the dictyosomes themselves (Robinson and Ray, 1973).

Plasmolysis and osmotic shock have also been shown to affect membranes by depolarization and selective modification of uptake sites. Racusen et al. (1977) found that plasmolysis of Elodea leaves with 0.7M mannitol resulted in a depolarization of the membrane such that the normally large negative membrane potential was replaced by a small positive potential. The effect was reversed upon deplasmolysis suggesting that turgor pressure influences the membrane potential of the plant cell. The very short osmotic shock treatments with 0.6M mannitol which induced branching in Stigeoclonium would be consistent with an involvement of membrane depolarization in branch induction for Stigeoclonium. Furthermore the fact that branching is induced by temporary plasmolytic treatments above incipient plasmolysis (0.4M mannitol) would be consistent with a membrane depolarization at this concentration, but not at 0.2M. Such a depolarization at 0.7M but not at 0.3M mannitol was observed by Racusen et al. (1977) for Elodea cells. As well as affecting the ionic composition of cells by interfering with membrane properties, plasmolysis may cause changes in the properties of membrane proteins per se. Studies on bacterial systems have shown that osmotic shock with 0.5M sucrose is a non-destructive method of selectively modifying events at the plasma membrane (Heppel, 1967; Neu and Heppel, 1965).

Osmotic shock markedly inhibits uptake of some substances and protein is released primarily from the bacterial periplasmic space (Heppel, 1967). In many cases (Anraku, 1968; Wilson and Holden, 1969; Wiley, 1970; Kalckar, 1971; Berger and Heppel, 1972; Aksamit and Koshland, 1972; Willis and Furlong, 1974) a protein ligand was isolated

from the external osmoticum which would bind with the substance whose uptake was inhibited. Uptake activity could be partially or totally restored by adding back components of the shock fluid (Anraku, 1968; Wilson and Holden, 1969; Hazelbauer and Adler, 1971; Kalckar, 1971). Thus the proteins released during brief osmotic shock of bacteria are probably important constitutents of their uptake mechanism (Oxender, 1972) and most interestingly have also been implicated in the chemotactic process (Hazelbauer and Adler, 1971). Recent work on plant systems reveals similar phenomena. Osmotic shock of oat coleoptiles inhibited the uptake of amino acids such as ∝- aminoisobutyric acid, 3-0 methyl glucose and leucine but had little or no effect on respiration or protein synthesis. Measurements of respiration indicated that energy production was not seriously affected by osmotic shock. Osmotic shock led to a depolarization of the membrane of many cells, and an efflux of ∝-aminoisobutyric acid and $K^+$ (Rubinstein et al. 1977). It was concluded that osmotic shock selectively modifies the plasma membrane, possibly by removal or inactivation of sites of uptake and a depolarization of the membrane. Thus although the effect of plasmolysis on plant cells probably involves an intricate chain of events it is likely that the integrity of the plasma membrane is central to the induction of branching by plasmolysis in Stigeoclonium.

## Prolonged plasmolysis

The results of these experiments suggest an inability of the nucleus and associated cytoplasm to move towards one end of the cell under prolonged incipient plasmolysis. The results would also indicate some role of the vacuoles in this movement of the nucleus and associated

cytoplasm preceding branch initiation. It is possible that the vacuoles have a mechanical, physiological role in forcing the nucleate cytoplasm towards various areas of the cell during branch initiation. Under conditions of incipient plasmolysis it is suggested that the vacuoles cannot undergo the contraction and expansion that would be necessary to form one large vacuole at the end of the cell and are thus maintained in their normal state as two vacuoles one at either end of the cell. As such the vacuoles are prevented from providing any differential pressure across the cell and so the nucleus cannot be moved.

These results on branch polarity in Stigeoclonium have some interesting parallels with studies on polar embryo formation in the brown alga Fucus (Torrey and Galun, 1970). When Fucus zygotes were grown in a hypertonic medium of seawater-sucrose and unilaterally illuminated they formed apolar embryos. The sucrose was considered to prevent the expression of polarity by preventing the establishment of a pressure potential across the embryo necessary for rhizoid elongation. Further evidence for a role of vacuoles in developmental processes comes from studies on budding in yeasts (Matile and Wiemken, 1976). During the extrusion and enlargement of buds in yeast there is an enlargement of vacuoles for turgor production. In the green alga Oedogonium the explosive process of ring splitting during cell elongation has been attributed to a build up of turgor pressure of the vacuole possibly through a discharge of large golgi-derived vesicles into the vacuole (Pickett-Heaps and Fowke, 1969).

## Effect of Plasmolysis during the cell cycle

The effect of the time of plasmolysis on the subsequent branch patterns is largely determined by its interaction with the time of mitosis and cytokinesis. (see Fig. 2.16 and Fig. 3.7) If cells are plasmolysed before cytokinesis, most cells complete cytokinesis before initiating branches. If plasmolysis occurs after cytokinesis, immediately adjacent cells will produce branches without further division. These studies clearly illustrate the dynamic interaction between cell division and branch initiation which characterises branching patterns in Stigeoclonium.

Cells may be more sensitive to the deleterious effects of plasmolysis at different times during the cell cycle. Studies on the dehydration of seeds (Brunori, 1967; D'Amato, 1972) have shown the DNA synthesis or S phase of the cell cycle to be the most sensitive to dehydration. It is possible that this sensitivity may be related to changes in the permeability of the plasma-membrane during the cell cycle since studies on Chinese hamster ovary cells found an increase in the permeability of the plasma-membrane to fluorescein during the early part of the S phase (Cercek et al., 1973).

In these studies, plasmolysis during mitosis and cytokinesis resulted in considerable cell death. This may also be due to changes in membrane permeability, possibly to calcium ions.

In Mougeotia light increases the flux of calcium ions across the plasma-membrane (Wagner and Bellini, 1976) while for many cells calcium increases during cell division (Berridge, 1976). As discussed in the last

chapter, plasmolysis may also induce an increased calcium influx across the plasma-membrane. The combination of those three events (light, cell division and plasmolysis) all leading to an influx of calcium could result in toxic levels of calcium in the cells.

## Centrifugation

The effects of acropetal centrifugation of Stigeoclonium cells are most likely explained by a displacement of the nucleus and associated cytoplasm towards the apex of the cell possibly involving some breakage of plasmodesmatal connections at the base of each individual cell. Although the protoplast may become retracted from the basal end wall during centrifugation it was observed to reposition itself against both end cell walls after centrifugation. It is most likely that the protoplast expands to re-establish contact with the basal cell wall immediately after centrifugation ceases.

Centrifugation has been used to change the axis of developmental polarity in both plants and animals. The polarity of growth in the protonema of Pteris vittata can be altered by centrifugation (Nakazawa, 1963; Ootaki, 1963). Centrifugation, leading to a movement of plastids and nucleus to the base of the apical cell, resulted in a branch being formed at the base of the cell and sometimes a rhizoid at the apex. In the studies of Ootaki (1963) reversal of polarity was obtained when apical cells longer than 300μ were centrifuged for 15 minutes at forces greater than 5,000 Xg. When the cell was longer than 300μ, the stratification caused by centrifugation was maintained, possibly due to the large vacuole which accumulated at the apex of the cell.

Thus, the ability to change the axis of developmental polarity in many cases may depend on the maintenance of cytoplasmic stratification caused by centrifugation. For example, Czaja (1930) could change the polarity of Cladophora but only after repeated centrifugations. Studies

by Whitaker (1937) on the Fucus egg showed that the developmental polarity could be determined by the axis of centrifugation. Centrifugation of Fucus eggs at 3,000 Xg for 15-20 minutes resulted in rhizoid formation at the centrifugal pole. In a small proportion of eggs centrifugation did not determine the position of rhizoid formation and this was correlated with redistribution of the cell organelles immediately after centrifugation. More recently, (Schmiedel and Schnepf, 1979b; Schmiedel and Schnepf, 1980) polarity in the moss Funaria hygrometrica was reversed by centrifugation especially when treatment with colchicine prevented the remigration of the nucleus.

Several cases in which the axis of developmental polarity could not be changed by centrifugation, e.g. studies on Equisetum (Mosebach, 1943; Nakazawa, 1957), Vaucheria spores (Weber, 1958) and on Coccophora and Sargassum eggs (Nakazawa, 1961) may be due to an ability of the stratified cytoplasm to reorganise immediately after centrifugation. Other examples are thoseof animal eggs such as Crepidula (Conklin, 1917) and Urechis eggs (Pease, 1939) in which developmental polarity could not be changed by centrifugation.

## Combined plasmolysis and centrifugation

The small number of cases where two branches of opposite polarity were produced from opposite ends of an individual cell by combined plasmolysis and centrifugation, illustrate the importance of the position of the nucleus and associated cytoplasm for the site of branch initiation.

The results also suggest that there may be an intracellular gradient of polarity which the nucleus responds to depending on its position within the cell to produce branches of opposite polarity at opposite ends of a single cell.

## Cutting

The demonstration of polarity in plants has most commonly been achieved through the polarity of regeneration after removal of a piece of the plant (Sinnott, 1960). The "cutting" experiments give a clear demonstration of the underlying polarity of the filament in Stigeoclonium. The fact that only the morphologically uppermost cell initiates a branch after cutting, shows that the correlative inhibition of branching is maintained even in fragments which do not contain an existing branch.

The apical cell may be the source of an inhibitor to both branch and rhizoid initiation. The apical fragments observed in these surgical experiments never produced branches, suggesting that the apical cell inhibited branch development. The reduction in frequency of rhizoid initiation from short apical fragments containing less than four cells (not including the apical cell) could best be explained if the apical cell was also regarded as the source of an inhibitor to rhizoid initiation. However since no branches were formed in these apical fragments the threshold of inhibitor allowing initiation of branches would have to be lower than that allowing rhizoid initiation.

Laser microbeam irradiation

The laser results also reveal the underlying polarity of the filament. If one considers branching to be regulated by an inhibitor, the evidence from these laser experiments would indicate that the inhibitor must be transmitted from the apex towards the base of the plant.

The observation that laser shots induced the initiation of branches in cells immediately above a branch point but not below the branch point reveals a strong polarity in the correlative inhibition of branching.

The lasing results would also be consistent with the hypothesis that inhibition of double branch points may be due to a combined inhibitory effect of the apical cell of the first branch and of other apical cells in branches immediately above. The filament forming the main axis would provide a central channel for the inhibitory signals from the apical cells of branches. An interruption to this central channel immediately above a single branch point results in the formation of a double branch point.

The mature Stigeoclonium plant may comprise a number of overlapping inhibitory fields with sources at the apical cells. The basal cell of a branch could receive inhibitory signals from both the apical cell of the branch it bears as well as from the apical cells of branches above it via symplastic transport along the main

filament. A secondary branch point could only be formed if the combination of both these inhibitory signals was lower than a critical threshold level for branch initiation. The laser studies also suggest that rhizoid initiation may be inhibited by apical cells.

The isolation of short files of cells resulted in branch initiation from the cell nearest the apex but no rhizoid initiation from the basal end of the file. In this case the more rapid initiation of a branch at the apical end may inhibit subsequent rhizoid development.

However lasing of single cells remote from branch points always resulted in rhizoid initiation from the acropetally adjacent living cells. The induction of rhizoids close to mature branch points and even from the same basal cell which bore a mature branch could still be consistent with apical cells as a source of an inhibitor to rhizoid development. In both cases, rhizoids were formed only near, or from, branch points with the apical cell quite distant from the branch point.

## 3.4 DISCUSSION OF RESULTS

(continued)

### 3.4.2 Drug Treatments

#### 1. Colchicine and cytochalasin B

Most eukaryotic cells have a cytoskeleton comprising microfilaments and microtubules, which is correlated with many aspects of cell form and movement (Hepler and Palevitz, 1974). Several studies have also indicated that this cytoskeleton is involved in the intracellular movement and positioning of organelles. In this study, two drugs known to affect the cytoskeleton, colchicine (which affects the microtubules), and cytochalasin B (which is known to affect the microfilaments), were employed to study their effects on the polarity of branch regeneration.

Other workers have also shown a differential effect of cytochalasin B and colchicine on plant growth and development. Plant cells display two types of growth. There is an overall surface growth as in coleoptiles, hypocotyls, epicotyls and roots, and tip growth as in root hairs and pollen tubes. It has been reported by Sawhney and Srivastava (1977) that overall surface growth is colchicine - sensitive while tip growth of the root hairs of lettuce seedlings is inhibited by cytochalasin B.

In another study Mollenhauer and Morré (1976) found that cytochalasin B but not colchicine inhibited the migration of secretory vesicles in the root tips of maize. Treatment with cytochalasin B did

not seem to inhibit the functioning of the golgi apparatus but prevented the movement of the secretory vesicles from the dictyosomes to the sites of fusion at the cell surface. They concluded that a cytochalasin B-sensitive subcellular component, most probably a microfilament-like component, is involved in the vectorial movement of the secretory vesicles.

In other studies a differential effect of cytochalasin B and colchicine has been found on the traumatotactic movement of nuclei. A primary wound reaction in plant cells is the traumatotactic movement of the nuclei in cells adjacent to the wound (Miehe, 1901; Ritter, 1911). In the cell adjacent to the wound the nucleus first increases in volume and then migrates to a position within the cell which is closest to the wound. The mechanism of this traumatotactic movement has been studied in the Tradescantia leaf epidermis (Schnepf and von Traiteur, 1973; Schnepf and Volkmann, 1974; Schnepf and Klump, 1975). Studies have shown that the traumatotactic movement of the nuclei is sensitive to cytochalasin B but not colchicine and it has been suggested that microfilaments but not microtubules play a role in the motile apparatus (Schnepf and von Traiteur, 1973). Other studies with inhibitors (ethionine, puromycin and actinomycin D) have suggested that the process depends on the synthesis of new messenger RNA and new protein after wounding (Schnepf and Klump, 1975). Intercellular transport of information on wounding takes place by unknown mechanisms but the finding that the traumatotactic movement is not influenced by morphactin suggests that it is not transmitted through an auxin gradient (Schnepf and von Traiteur, 1973).

Any critical evaluation of drug treatments must include a proper assessment of side effects. The action of colchicine is attributed to a binding of colchicine to a pool of soluble sub-units which is in dynamic equilibrium with the polymerised cytoplasmic microtubules (Borisy and Taylor, 1967; Wilson and Friedkin, 1967). The formation of these complexes removes the soluble sub-units from dynamic equilibrium with polymerised units and results in depolymerisation of microtubules. A critical review of colchicine and plant microtubules (Hart and Sabnis, 1976) has cautioned that the relatively high concentration of colchicine needed to depolymerise plant microtubules can also affect other cellular processes, particularly those associated with the plasma-membrane.

0.2% colchicine, which normally depolymerises plant microtubules, has no effect on the polarity of branch initiation. The ultrastructural studies (Fig 2.25) only rarely show microtubules during branch initiation in conventionally fixed material. These studies, however, do not preclude the possibility that side effects were also produced, in fact, the abnormal shape of the branch initial might reflect some possible side effect.

By contrast the cytochalasins (Greek cytos cell, chalasis relaxation) are a group of fungal metabolites which inhibit a variety of cellular movements such as cytoplasmic streaming, cytokinesis and pinocytosis as well as the transport of sugars (Wessells et al. 1971; Carter, 1972; Pollard and Weihing, 1974). To explain these effects it has been suggested that the cytochalasins act on the cortical microfilaments

and thus alter the integrity of the contractile apparatus of the cell (Wessells et al. 1971).

Although they differ in potency, cytochalasin D and cytochalasin B produce similar cytologic effects (Miranda et al. 1974; Wessells et al. 1971; Carter, 1972). While cytochalasin B inhibits hexose sugar transport in many cells (Mizel and Wilson, 1972; Kletzien and Perdue, 1973) recent studies using tritiated cytochalasins (Tannenbaum et al. 1975, 1977a,b) indicate that cytochalasin B and cytochalasin D possess different high affinity binding sites but share a common low affinity binding site. The high affinity binding site for cytochalasin D, which does not bind cytochalasin B, appears to be a peripheral protein located on the cytoplasmic face of the plasma membrane and is most likely plasma membrane-associated myosin (Tannenbaum et al. 1977). The high affinity binding site for cytochalasin B, which does not bind cytochalasin D, appears to be an integral membrane protein associated with hexose transport (Lin and Spudich, 1974a,b). The low affinity site common to cytochalasin D and cytochalasin B which may be actomyosin probably affects contractility-related phenomena (Tannenbaum et al. 1977a,b), in agreement with earlier suggestions on the action of cytochalasin B (Lin et al, 1974; Lin and Spudich, 1974c).

The initiation of apolar branches when plasmolysis is followed by cytochalasin B treatment would suggest that microfilaments are necessary for the expression of branch polarity. However the abnormal shape of some of these branches suggests side effects.

## Caffeine

Caffeine and other xanthic bases inhibit cytokinesis by preventing the arrangement and fusion of the Golgi vesicles which form the cell plate (Lopéz-Saéz et al. 1966; Paul and Goff, 1973; Pickett-Heaps , 1969). The blockage of cytokinesis by pulse-treatments with caffeine has been used to induce a population of binucleate cells characterized by a synchronous development of the cell cycle (Becerra and Lopéz-Saéz, 1978).

At the molecular level, the mode of action of caffeine is still being elucidated. Paul and Goff (1973) proposed that caffeine interfered with cytokinesis by affecting a process of membrane recognition and/or fusion in which calcium was essential. Studies on the sarcoplasmic reticulum support the concept that caffeine displaces calcium from membrane binding sites but the mechanism of this displacement is not clear (Weber, 1968).

In higher plants the binucleate cells produced by caffeine treatment remain binucleate during a recovery period, i.e. cytokinesis does not ensue after a caffeine induced delay (Becerra and Lopéz-Saéz, 1978). However in various green algae caffeine treatment produces abnormal cell divisions (Hinz, 1974).

In most cases, for Stigeoclonium the binucleate cells produced by caffeine treatment underwent cytokinesis during the recovery period and the nuclei were partitioned into small cells. Plasmolysis induced branch formation adjacent to each nucleus.

Occasionally cytokinesis did not ensue and the large bulges formed from the side of these cells after plasmolysis, subsequently produced two branches, i.e., one branch per nucleus. Occasionally plasmolysis induced two branches to be formed directly from a single binucleate cell which did not undergo cytokinesis after caffeine treatment. Again it can be concluded that each nucleus of the binucleate cell could be induced to initiate a single branch even without an ensuing cell division.

The position of branch initiation following caffeine treatment can be correlated with the position of the nucleus at the time of plasmolysis. The branches are initiated at the centre of the original binucleate cell, and after cytokinesis this is seen as branching from the apex of one cell and the base of the adjoining cell. In most cases these branches are not strongly polar in that the branches are formed at approximately right angles to the main filament.

## Actinomycin D and cycloheximide

Actinomycin D is known to inhibit the DNA-dependent synthesis of mRNA (Goldberg and Rabinowitz, 1962) while cycloheximide inhibits protein synthesis by affecting 80s ribosomes (Ennis and Lublin, 1964).

Classical studies on nuclear-cytoplasmic interactions have been undertaken on the alga Acetabularia using inhibitors of mRNA and protein synthesis (Hämmerling, 1963; Brachet, 1968). These studies have shown that morphogenesis in Acetabularia is regulated at the translation stage of protein synthesis using pre-formed, long-lived mRNA. Although morphogenesis in Acetabularia is dependent on the transcription of nuclear DNA into stable, long-lived mRNA, the time of expression of the genetic information is controlled by the cytoplasm.

In the case of the plasmolytic induction of branching in Stigeoclonium, de novo mRNA and protein may be necessary for branch extension since treatment of the cells with actinomycin D and cycloheximide immediately after plasmolysis inhibited branch initiation. However upon subsequent release from the inhibitor (actinomycin D) the cells produced branches in similar numbers to cultures plasmolysed without actinomycin D treatment. It is possible in these studies that mRNA is present at the time of plasmolysis for some very early event in branch initiation or determination, but the full morphological expression of branching requires de novo mRNA synthesis. In Blastocladiella, the early stages of zoospore germination are not inhibited by actinomycin D or cycloheximide but later stages are (Truesdell and Cantino, 1971; Leaver and Lovett, 1974).

## 5-Aminouracil

Mitotic synchronization of onion root cells by 5-aminouracil (a thymine analogue) was attributed to accumulation of cells in the final portion of the S phase of interphase (Diéz et al. 1976). The passage of cells through previous stages of mitosis was not affected by 5-aminouracil. The results suggested the existence of a particular period located in late interphase which appears to be highly sensitive to 5-aminouracil. Removal of the drug frees the blockage and the accumulated cells synchronously complete interphase, making up the mitotic wave.

A coupling between the S phase and the development of cell competence to respond to signals to differentiate has been proposed by Holtzer (1963). During the S phase the DNA is stripped of its protein coat (histone and acidic protein) (Kishimoto and Lieberman, 1964), and this may allow changes in the stability of the differentiated state so that a cell can enter a new pathway of differentiation.

The uniform branching response in Stigeoclonium after treatment with 5-aminouracil may be due to synchronization of the cells at a stage in the cell cycle which is sensitive to signals for a change in the cell state towards differentiation.

## 3.4 DISCUSSION OF RESULTS

(Continued)

### 3.4.3 Intercellular communication in Stigeoclonium

#### 1. Ultrastructure of plasmodesmata after plasmolysis.

A study of plasmodesmata between synchronously and asynchronously developing cells of the antheridial filaments of Chara vulgaris found that plasmodesmata between asynchronously developing cells were plugged by an electron dense homogeneous material (Kwiatkowska and Maszewski, 1976). It was proposed that plugging of the plasmodesmata inhibited the symplastic transport of a "synchronizing principle." The plugging of plasmodesmata with electron-dense material has also been found in the lateral walls of root cells (Burgess, 1971) and oat coleoptile cells (O'Brien and Thimann, 1967). In the filamentous green alga Bulbochaete hiloensis (Fraser and Gunning, 1969) dark staining material was found in the cell wall.

A darkening of cell walls is one of the ultrastructural characteristics of chilling injury noted for grapefruit (Platt-Aloia and Thomson, 1976) and tomato cotyledons (Ilker et al. 1976). The darkening of the cell walls was similar to that found during senescence of grapefruit rind (Platt-Aloia and Thomson, 1976) and was considered to be a stress response accompanied by callose deposition during chilling of tomato cotyledons (Ilker et al. 1976).

It is possible that the electron dense material seen in the

cross walls of Stigeoclonium after plasmolysis and during senescence is the same as that found in the cases discussed above, but confirmation of this would require an identification of the material.

## 2. Callose deposition after plasmolysis

Callose is a β, 1-3 glucan which fluoresces yellow when stained with aniline blue (Aspinall and Kessler, 1957; Eschrich and Currier, 1964). It occurs widely in plants (Eschrich, 1956) including phloem (Currier and Strugger, 1956; Eschrich, 1956; Currier, 1957), new cell walls and developing cell plates (Currier, 1957; Waterkeyn, 1967; Fulcher et al. 1976) and pit fields(Currier and Strugger, 1956). It is most commonly detected in plant cells under some form of stress such as mechanical damage (Currier and Strugger, 1956; Currier and Webster, 1964; De Kazos and Worley, 1967), plasmolysis (Currier and Strugger, 1956; Eschrich, 1957), viral infection (Wu and Dimatan, 1970; Shimomura and Dijkstra, 1975) fungal infections (Aist, 1976; Garciá-Arenal and Sagasta, 1977) and temperature stress (Smith and McCully, 1977). This has led to the hypothesis that callose is formed to seal off damaged areas and to protect the cell during stress (Eschrich, 1956).

### Deposition of Callose

In a study of pollen tubes Cresti and van Went (1976) found two distinct sites of callose deposition. Callose deposition was detected as a distinct layer outside the plasma membrane as well as within the cytoplasm as large callose plugs. It was proposed that the layer of callose outside the membrane may be involved in binding of water by

imbibition as was first suggested by Eschrich (1965) for callose in sieve elements and may play a role in maintaining osmotic conditions in the pollen tube. The large callose plugs were correlated with a disorganisation and disintegration of areas of the older cytoplasm and the plugs may function to seal off these areas.

## Synthesis of Callose

The mechanism of synthesis of callose is still unknown. In sieve tubes, UDP-glucose is the immediate precursor of callose (Eschrich et al. 1972) and the rapid formation of callose within minutes of wounding suggests that the enzymes necessary for callose synthesis may be continually maintained in strategic positions within the phloem tissue (Evert and Derr, 1964). The studies of Helsper et al. (1977) on pollen tubes of Petunia hybrida have shown some β, 1-3 glucan synethethase activity in the golgi vesicles. It is possible that golgi vesicles have some function in formation of callose after fusion with the cell membrane.

## Specificity of Aniline Blue Staining

The use of aniline blue to detect callose was based on the staining of isolated sieve plates from Vitis vinifera which are known to be composed largely of β, 1-3 glucan (Aspinall and Kessler, 1957; Kessler, 1958; Eschrich and Currier, 1964). Therefore, as Smith and McCully (1978) have said, "absolute chemical definition, in one instance, of the substance induced to fluoresce with aniline blue does not necessarily make the general rule without exception, although the localisation of intense aniline blue fluorescence in specific regions

such as new cell walls, sieve plates and pit fields does indicate some common property." A reinvestigation of the histochemistry of aniline blue-induced fluorescence concluded that it does not specifically indicate β, 1-3 glucans (Smith and McCully, 1978). The apparently selective staining may be based on a more open wall construction which would give the fluorochrome more ready access to polysaccharide binding sites in traditional callose regions such as sieve plates, pit fields and new cell walls (Smith and McCully, 1978). Thus, the validity of aniline blue fluorescence as a means of detecting the occurrence of β, 1-3 glucans in various plant species is questionable. However, there is possibly a stronger case for its use in detecting stress reactions in an individual plant. The work of Smith and McCully (1977) on the effects of mild temperature stress showed an unequivocal callose reaction detected with aniline blue.

The deposition of callose during plasmolysis in Stigeoclonium would be consistent with a stress reaction which may block intercellular communication. The degree to which callose can prevent the transport of molecules or ions between plant cells is unknown and, in particular, its ability to block ion transport from cell to cell is not at all established.

## 3. The role of calcium in intercellular communication in Stigeoclonium

It has been well established that calcium has a central role in the regulation of intercellular communication in animal cells. Loewenstein(1968) has elegantly demonstrated that uncoupling occurs in Chironomus salivary gland cells when an elevated calcium level occurs near the gap junctions.

The role of calcium in regulating intercellular communication in plant cells is virtually unexplored. The studies reported here on Stigeoclonium suggest that calcium may have a central role in controlling intercellular communication also in plant cells. The chelating of cellular calcium with EDTA prevented the formation of callose near the cross walls of Stigeoclonium and the EDAX studies show the presence of calcium in the region of the callose deposit (i.e., not calcium pectate in the middle lamella).

A role for calcium in controlling polarity in plant development could also be suggested on the basis of studies using the calcium ionophore A 23187 and a calcium free medium. The ionophore A 23187 has been shown to mediate the passage across membranes of calcium ions (Reed and Lardy, 1972). The resultant influx of calcium ions may disrupt any ionic gradient associated with the cell cortex and eventually result in changes in the polarity of cell differentiation (Jaffe et al. 1974; Jaffe and Nuccitelli, 1977). Recently such changes have been reported after treatment of the alga Acetabularia with the calcium

ionophore (Goodwin and Pateromichelakis, 1979) which reversibly inhibited the formation of apical structures (whorls and caps). In the studies reported here on Stigeoclonium, treatment with the calcium ionophore resulted in changes in branch polarity such that markedly fewer branches were formed towards the apex of cells.

During ionophore treatment, many branches were formed as stunted initials at the base of the cells but the frequency of branching was not markedly different from untreated controls. Upon release from the ionophore treatment, branching frequency was markedly increased but with a similar polarity of branching as observed during ionophore treatment. The results suggest that treatment with the ionophore induces a branching response but may inhibit subsequent development of the branches. This would be consistent with the stunted branch initials formed during ionophore treatment and may be due to an inhibitory effect of high intracellular calcium levels on a process necessary for branch extension.

No marked callose deposition was noted during ionophore treatment and this would suggest that increases in intracellular calcium per se do not initiate callose formation. The formation of callose during plasmolysis may be as a reaction to environmental stress but its deposition at specific sites in the cell such as near the cross walls may require free calcium.

The source of calcium used for triggering changes in cell development can be either intracellular or extracellular (Baker, 1976). Under circumstances where cells are maintained in calcium-free solutions it is likely that mictochondria release calcium to maintain the cytoplasmic free-calcium levels (Borle and Anderson, 1976). This release of calcium may

also disrupt intercellular communication and ionic gradients associated with the cell cortex and thereby result in changes in the position and polarity of development as demonstrated for branching in Stigeoclonium.

## 3.5 GENERAL DISCUSSION ON BRANCH PATTERNS IN STIGEOCLONIUM

### 3.5.1 The position of branches

Branching in Stigeoclonium is an example of a broad class of patterns called spacing patterns. Examples of spacing patterns include heterocyst spacing in blue-green algae, bristle patterns in the insect epidermis, phyllotaxis and stomatal patterns in leaves.. These topics are reviewed briefly below, following which their relevance to branch patterns in Stigeoclonium is discussed.

#### (a) Blue-green algae

The filamentous blue-green algae are procaryotes which form specialized cells called heterocysts at regular intervals along the vegetative filament (Fritsch, 1951). The organism provides a simple, one-dimensional pattern of heterocysts which can readily be used to study the spatial control of differentiation. The heterocysts of Anabaena are thick-walled, highly-differentiated cells which go through a well-defined sequence of ultrastructural changes during differentiation (Lang and Fay, 1971; Wilcox et al. 1973 b ).All the vegetative cells of Anabaena are connected by microplasmodesmata but the mature heterocyst develops a 'plug' of material in the neck of the junction with its adjacent vegetative cells (Wilcox et al. 1973 b ). Each of the vegetative cells divides asymmetrically but the heterocyst does not divide (Mitchison and Wilcox, 1972). Heterocysts develop from the smaller daughter cell of an asymmetric division (Mitchison and Wilcox, 1972).

Heterocysts are involved in the nitrogen-fixing process and are possibly the sole sites of nitrogenase, at least under aerobic conditions (Fleming and Haselkorn, 1973; Stewart, 1973).

The pattern of spacing for heterocysts has been studied by Wilcox et al. (1973 a and b) and Wolk (1975). The studies of Wilcox et al. (1973 a and b) led to a model of pattern formation in Anabaena which combined an inhibitory zone/threshold mechanism with asymmetric division and competition between proheterocysts.

It was first suggested by Fogg (1949) and later by Wolk (1967) that heterocysts had inhibitory zones extending from them so that new heterocysts developed only from cells beyond the inhibitory zone of existing heterocysts. Thus a diffusional gradient of inhibitor is said to be established around each heterocyst and only cells in which the concentration of inhibitor falls below a critical threshold level could begin to differentiate into heterocysts. Mitchison and Wilcox (1972) have modified this inhibitor/threshold postulate to include the asymmetric division of cells, such that a "new heterocyst will only develop from the first smaller daughter cell to arise by division outside the inhibitory zone." A further mechanism for limiting the number of potential heterocysts in a subthreshold region is based on competitive interactions (Wilcox et al. 1973 a and b).

By studying the regression of proheterocysts (prospective heterocysts) Wilcox et al. (1973b) concluded that the heterocyst spacing was based on an underlying competitive mechanism. If filaments of Anabaena are broken near a proheterocyst, the proheterocyst can reverse

its development and become a vegetative cell. This was interpreted as a dependence of the proheterocyst upon neighbouring vegetative cells for removal of an inhibitor. It was postulated that the proheterocyst is both a source of the inhibitor and susceptible to the inhibitor during its early development. Thus if two proheterocysts begin to develop close together each will contribute to an increase in the level of inhibitor in the other and only one of the competing proheterocysts will be able to develop. The model can be represented by the following diagram from Wilcox et al. (1973a).


X is a non-diffusible substance whose concentration expresses the state of heterocyst development.

Y is a diffusible inhibitor of heterocyst differentiation.

Y is produced at a rate (i) dependent on the concentration of (X).

The synthesis of X has an autocatalytic component (ii).

Y inhibits the synthesis of X (iii).

The model can elegantly explain why only the smaller daughter of a cell division should produce a heterocyst if one assumes that the efficiency of a cell as a source of inhibitor is an increasing function of its size. Thus a smaller cell would have an advantage over other cells since it would produce relatively less inhibitor (Y) for a given cell state (expressed as a concentration of X).

Wolk (1975) expressed doubts about the autoinhibition of heterocyst spacing proposed by Wilcox et al. (1973 a and b). He believed that the regression of proheterocysts following the cutting of the filament near them may be a non-specific wound response. Wilcox et al. (1975) argued against the effects of a wound response by showing that despite the wounding involved in forming an "artificial end" the distribution of new proheterocysts was the same as that for "normal end" filaments. Furthermore it was found that the tryptophan analogue 7-azatryptophan which affects the spacing of heterocysts (Mitchison and Wilcox, 1973), also affects the differentiation process so that proheterocysts can subsequently develop close to a break where they would usually regress (Wilcox et al. 1975). This would tend to argue against a non-specific wound affect.

Modifications of the heterocyst spacing pattern such as that produced by 7-azatryptophan (Mitchison and Wilcox, 1973) are obtained also with an inhibitor of transcription, rifampicin (Wolk, 1975). Both compounds produce a reduction in the mean heterocyst spacing but they appear to have different sites of action during the development of the heterocyst (Wilcox et al. 1975). While 7-azatryptophan reduces, rifampicin increases, the probability of regression of proheterocysts in isolated fragments (Wilcox et al. 1975). Both 7-azatryptophan and rifampicin are considered to decrease the extent of the inhibitory zones around existing heterocysts but their exact mode of action on the underlying biochemistry of pattern formation is unknown.

(b) Bristle Patterns in the Insect epidermis

The insect epidermis provides a rich source of markers for studying the spatial control of cell differentiation. A simple two dimensional spacing pattern is provided by the bristles on the abdominal cuticles of insect larva such as Rhodnius and Oncopeltus. To explain the bristle patterns observed in Rhodnius, Wigglesworth (1940) postulated that existing bristles inhibited the development of new bristles in their immediate vicinity by absorbing a diffusible substance produced by the neighbouring epidermal cells. A new bristle could only be formed in an area outside the field of depletion which contained a high enough concentration of the transforming substance.

Lawrence (1966a,b,c) has studied the development of both hairs and bristles in Oncopeltus. He postulated an inhibitory field around each bristle. To generate the observed patterns of bristles and hairs, using a computer, the inhibitory fields must be assumed to have a normal variation in size and in fact the field size must shrink while the hairs are being added (Lawrence, 1970).

Lawrence (1970) proposed that a "small group of epidermal cells sponsor the appearance of a new organ in their centre and in this view the developmental state of the bristles itself becomes irrelevant to the pattern forming process." He envisages pattern formation as due to a series of independent gradient systems which are interpreted by the epidermal cells; an hypothesis similar to the positional information concept of Wolpert (1969). Lawrence (1969) believed that "differences between competences of cells are, therefore, mostly responsible for the

structural heterogeneity."

(c) Phyllotaxis

The arrangement of leaf primordia at the shoot apex (phyllotaxis) has long intrigued botanists and mathematicians. The arrangement of leaves is usually highly regular and characteristic for a given plant species. Spiral arrangements are very common but other arrangements include alternate, opposite and whorled. Several theories to account for phyllotaxis have been advanced. These include a mechanical pressure hypothesis (Schwendener, 1878; Adler, 1974), a "first available space" hypothesis (van Iterson, 1907; Snow and Snow, 1962) inhibitor-diffusion hypotheses (Schoute, 1913; Richards and Schwabe, 1969) and the foliar helix hypothesis of Plantefol (1946).

Some experimental evidence for an inhibitor hypothesis is provided by the work of Wardlaw (1949) on the fern apex. Isolation of a leaf initial by deep radial incisions resulted in the isolated initial growing more rapidly, presumably because it was released from the inhibitory effects of neighbouring primordia or the shoot apex itself. Furthermore destruction of the site at which an initial was due to arise resulted in a shift towards this site of an initial which subsequently developed. This was thought to be due to the lack of any inhibition from the primordium site which had been destroyed.

A computer simulation of phyllotactic patterns based on an inhibitor-diffusion mechanism was successful in generating most of the phyllotactic patterns observed in nature (Veen and Lindenmayer, 1977).

Thus phyllotaxis could be regarded as a spacing pattern maintained by an inhibitory field.

(d) Patterns of stomatal distribution

The distribution of stomata on the surface of leaves is another example of a spacing pattern. The pattern is not random and in many cases stomata maintain a certain minimum distance between one another.

It has been suggested that the stomatal patterns could result from an inhibitory influence of each stoma on the initiation of further stomatal complexes in adjacent cells (Bünning, 1948, 1965; Bünning and Sagromsky, 1948). An alternative explanation is based on determinate cell lineages which produce stomata in only limited numbers of cells. This hypothesis is favoured by Sachs who believes that inhibition of stomatal development plays a minor role and at most extends to a distance of one epidermal cell (Sachs, 1974; Marx and Sachs, 1977; Sachs, 1978).

Resolution of this point will probably depend on experimental studies to investigate the effect of interrupting cell communication on any possible interaction between stomata.

(c) Comparisons with Stigeoclonium

Despite the obvious differences between Stigeoclonium, a eucaryote and Anabaena, a procaryote, there are certain parallels which can be drawn from a study of their patterning. Both organisms consist of single files of cells some of which, in Stigeoclonium, give rise to

branches while in Anabaena some cells differentiate to heterocysts. In both Anabaena and Stigeoclonium the differentiation process involves unequal divisions to produce heterocysts and branches respectively. In both organisms, pre-differentiation events include equal intercalary divisions into equivalent daughter cells. One difference is that the cells in Anabaena are connected by microplasmodesmata whereas those of Stigeoclonium are connected by true plasmodesmata similar to those found in higher plants. The structure and function of microplasmodesmata are less well-known than those of true plasmodesmata.

Also, in Stigeoclonium no reversion of branching has been observed and this is in contrast to the reversion which follows in Anabaena if the filament is cut near the proheterocyst. In both systems the pattern formation can be most readily explained by assuming the transcellular transport of an inhibitor arising from differentiating cells. This is discussed more fully for Stigeoclonium in the next chapter.

In the case of phyllotaxis and stomata patterns the differentiation is confined to specific regions of the organism; the shoot apex and leaf epidermis respectively. These two systems are in strong contrast with Stigeoclonium in which the differentiation and pattern formation of the branches occurs throughout the whole organism. The patterning in Stigeoclonium resembles more the pattern of distribution of hair cells in the insect epidermis.

In both Stigeoclonium and the insect epidermis the differentiated cells are used as local markers of polarity. The field of independent

inhibitory gradients postulated for the insect epidermis may be applicable to Stigeoclonium. Each apical cell in Stigeoclonium could be considered the source of an inhibitor to branching. Thus the mature plant could consist of a number of independent but overlapping fields with their sources at the apical cells. The inhibitory field in Stigeoclonium is considered to be polar in that it is directed from the apical cells to the base of the plant. The primary branching patterns in young filaments, recorded in Chapter 2, suggested some regulation of the branching pattern to maintain an optimal spacing between branches through a dynamic equilibrium of cell division and branch initiation. In this chapter regulation could be considered to follow disruption by plasmolysis since a spacing between the apical cells of the branches induced by plasmolysis, is established by the subsequent growth of the branch.

A detailed model for pattern formation in Stigeoclonium is discussed in the next chapter.

## 3.5.2 The polarity of branches

Extensive studies of the initiation and maintenance of polarity in plants (Bloch, 1965b) and animals (Nieuwkoop, 1977) have led to the views that the polarity of the cell resides in the cortical layer of the cytoplasm, namely the plasma-membrane and its associated structures. This conclusion is largely based on the indirect evidence that centrifugation leading to temporary stratification of cell contents does not lead to changes in cell polarity.

On more theoretical grounds the global properties of polarity are seen to be consistent with the cortical layer acting as a continuous layer throughout a multicellular system *via* a network of interconnecting channels (desmosomes in the animal embryo and plasmodesmata in the plant). Recently, Bluemink and Tertoolen (1978) have shown an anisotropic arrangement of plasma-membrane components (intra-membranous particles {IMP}) in the amphibian egg. A significant number of small IMP ($\leq$ 81 Å) were found only in the animal part of the amphibian egg. It is suggested that these local differences in IMP pattern may be a form of macromolecular "Braille" coding for positional information. The transmission of this localised membrane coding to the cytoplasm is supposed to be *via* the membrane-associated elements of the cytoskeleton, that is, microfilaments and microtubules.

At first there seems to be a conflict between the results of the isolation of cells resulting from the extirpation of adjacent cells by the laser and the prolonged "physiological" isolation of cells using plasmolysis. In one case, after prolonged plasmolysis, the cells appear

to lose their original polarity such that they regenerate branches in almost equal numbers towards the base or apex of the plant. By contrast, individual cells isolated by laser action retained their previous polarity. Although there is an apparent conflict here it may be resolved by looking at the mode of action of the two methods used to isolate the cells. In the case of prolonged plasmolysis there is a retraction of the plasma-membrane from the lateral cell wall. In an interesting study on regeneration of polarity in Bryopsis, Nakazawa (1975) also studied the effect of the retraction of the plasma-membrane from the cell wall. In his studies, the thallus of Bryopsis plumosa was separated into 5 mm and 2 mm protoplasmic fragments, arranged in a line inside the tubular cell. This state was obtained by pressing against the tubular thallus with a blunt glass needle at the appropriate interval. If the fragment was longer than 5 mm it regenerated a new thallus from what was originally its apical end. However, the original polarity was lost and regeneration occurred randomly in any fragment which was 2 mm or less in length. This loss of polarity in 2 mm fragments was attributed to a complete separation of the protoplast from the cell wall.

During prolonged plasmolysis of Stigeoclonium for 1 hour and 3 hours with 0.6M mannitol (Fig. 3.1 c and d) some retraction of the protoplast from the lateral walls was observed. However, following laser treatment and with cutting there is no retraction of the plasma-membrane from the lateral cell wall of the isolated cell and thus its existing polarity may not be interfered with. Alternatively, prolonged plasmolysis per se may affect the plasma-membrane, causing a loss of information on polarity.

The expression of branch polarity in Stigeoclonium may be dependent on the movement of cellular organelles. The cellular organelles provide an intracellular compartmentation in which the relative positioning of organelles is maintained after cell growth and division. To co-ordinate the cellular activities the movement and positioning of organelles must be under a high degree of control. Cytoplasmic movements have been classified into three basic types (Allen, 1974):

1. bulk cytoplasmic transport that accompanies pseudopodia formation and amoeboid locomotion,
2. bulk cytoplasmic transport that is unrelated to cell locomotion and serves to circulate protoplasm, that is cell streaming,
3. selective transport of cytoplasmic constituents, e.g. organelles like the nucleus, which is unrelated to any bulk transport.

It is considered that the endoplasm of cells is variably resistant to the displacement of organelles within it and behaves like a gel of cross-linked polymers undergoing sol⇌gel transformations (Allen, 1974).

Branching in Stigeoclonium may require both bulk cytoplasmic transport involving vacuoles and selective organelle transport involving microfilaments. The expansion of a single vacuole at one end of the cell during polar branch formation as well as the initiation of apolar branches between two terminal vacuoles at incipient plasmolysis suggests a role for bulk cytoplasmic transport by vacuoles during branch initiation. More selective transport of organelles such as the nucleus to the site of branch initiation may involve microfilaments. This is suggested by the changes in branch polarity after treatment with cytochalasin B.

The distinction between the determination and expression of branch

polarity allows an explanation for the changes in polarity of branching in Stigeoclonium produced by the various treatments discussed in this chapter.

Apolar branches arising from the centre of cells are formed if the expression of polarity is prevented by treatments which inhibit the movement of the nucleus and associated cytoplasm. As discussed above the effect of incipient plasmolysis and cytochalasin B may be explained on this basis as both treatments produce large numbers of apolar branches.

If the determinant of cell polarity resides in the plasma-membrane as a component that controls calcium influx into the cell, then the changes in polarity produced by the calcium ionophore A 23187 and prolonged plasmolysis could be explained. Prolonged plasmolysis may alter the distribution of the plasma-membrane component which determines polarity possibly, as discussed above, due to a retraction of the plasma-membrane from the lateral cell wall. The change in polarity of branches formed near dead cells after temporary plasmolysis may also be due to changes in the distribution of the component in the plasma-membrane which controls polarity. However it is also possible that these changes in polarity are associated with a traumatotactic movement of the nucleus towards the damaged cells which is a primary wound response in many plant cells (Miehe, 1901; Ritter, 1911). Treatment with the calcium ionophore may override any polar distribution of plasma-membrane components involved in calcium influx during branch initiation.

In both the prolonged plasmolysis and the calcium ionophore treatments few apolar branches are produced and branches are formed mainly from either

the base or apex of the cells. By contrast, treatments with incipient plasmolysis and cytochalasin B result in large numbers of apolar branches formed from the centre of cells.

This would suggest that the nucleus and associated cytoplasm is preferrentially moved in a longitudinal direction in the plane of the filament, possibly by the microfilaments, prior to branch initiation. Only when this movement in the plane of the filament is inhibited by maintaining cells at incipient plasmolysis or treating with cytochalasin B are large numbers of apolar branches produced.

The present studies on the polarity of branching in Stigeoclonium are consistent with the concept of the plasma-membrane as the repository of information on polarity but the expression of branch polarity may be controlled by the intercellular transport of a morphogen which binds to a plasma-membrane component that controls calcium influx into the cell. Calcium influx results in the movement of cellular organelles possibly via microfilaments to produce a polar branch. In this case the morphogen would act as an inhibitor of the expression of polarity with the plasma-membrane as the underlying determinant. Evidence for this hypothesis is discussed in detail in the next chapter.

# CHAPTER 4 : A MODEL OF PATTERN FORMATION

# CHAPTER 4 : A MODEL OF PATTERN FORMATION

In the first chapter the four basic patterns in nature were described and a number of models to explain their formation, largely based on animal development were discussed. In this chapter the universality of pattern formation in plants and animals is argued and a general model for pattern formation in Stigeoclonium is formulated, based on the descriptive and experimental studies in this thesis, and placed in the broader context of plant and animal development.

## 4.1 The universality of the process of pattern formation

In the plant kingdom, the patterns of multicellular development can also be divided into four basic categories (Bloch, 1965a):

1. Firstly, there is the axiate or branched pattern with apex-base polarity with the initiation of branches from the root and shoot,
2. Secondly, there are concentric or spiral patterns such as the cortex, stele in shoots and secondary thickening in dicotyledons,
3. Thirdly, there are radiate or explosion patterns such as primary phloem and xylem strands in the root, primary vascular strands and xylem rays in the dicotyledonous shoot,
4. Fourthly and finally, there are mosaic or meander patterns such as vascular strands in the monocotyledonous stem and stomatal patterns.

The process of pattern formation is controlled temporally and spatially and models have been made of so-called "clocks" and "maps" in developing organisms (see Goodwin, 1976). A number of models of pattern formation have been postulated on the basis of work with animal systems and were discussed in detail in Chapter One. At present the search is for a universal model of pattern formation which may apply to both plants and animals. A common underlying determinant of pattern formation may be present in both animals and plants but could be masked by differences in expression of the morphological patterns.

Graded distributions of developmental capacity, which would be consistent with Wolpert's Positional Information Theory, have been demonstrated in plants. The capacity of Acetabularia to regenerate a cap is dependent on the length and position of the piece of stalk. The results suggest that there is a gradient in the distribution of a cap-forming substance with the highest concentration in the stem region just below the cap (Hämmerling, 1963). There are apical-basal gradients in the rate of regeneration of isolated pieces of the liverwort Riella (Stange, 1957). Similar gradients in regenerative capacity have been found in the fern Pteris vittata demonstrating the inverse relationship between the age of the isolated cell and the time required to form the first protonema cell (Ito, 1962). In the hybrid moss Physcomitrium-Funaria, isolated pieces of the adult sporophyte form only a protonema. In young sporophytes, however, apically-and basally-isolated regions produce different regeneration products (Bauer, 1957).

The major difference in pattern formation between plants and animals at the phenomenological level may be the importance of unequal cell division in the development and expression of cellular patterns for plants. The plant cell is enclosed in a rigid cellulose cell wall which limits cell movement. Much of plant development depends on the regulation of orientation of the plane of cell division to create cellular patterns (Bloch, 1965a).

Unequal division marks the first step in the expression of cell differences at various stages in the plant life cycle. Where such division occurs in the zygote it results in cells which give rise to the two main parts of the plant, namely the thallus and the rhizoid in algae and other lower plants, the suspensor(s) and the embryo proper in higher plants. Other examples of unequal division occur in the final stages of development of organs, as in the formation of stomata and trichoblasts. The differentiation of root hair cells involves unequal cell division. Epidermal cells of the root divide unequally into a large and a small cell. The smaller cell, called a trichoblast, develops as a root hair (Sinnot and Bloch, 1939). Unequal divisions of the tetrahedral apical cell in mosses and ferns is in part the basis of cell patterning in these plants (Bünning, 1952; Bloch, 1965a). The apical meristem is surrounded by a single large tetrahedral cell which divides by unequal divisions giving rise to daughter cells from the three proximal faces in succession. The larger distal daughter cell (the residual apical cell) of each division retains its capacity for further division, whereas the smaller, inner cell is capable only of a small number of further divisions, giving rise to cell lineages which undergo differentiation.

The apical meristem of plants may correspond to a true positional field. A feature of many meristems is their capacity to regulate their own development (Ball, 1948; Sussex, 1952). These experiments demonstrate that the shoot apex of plants, particularly the promeristem region may be a developmental field with a population of unspecialized, rapidly dividing cells, any part of which (perhaps within minimum size limits) is capable of functioning like the whole promeristem.

Although unequal division is correlated with cell patterning in many plants, cytokinesis per se is not necessary for the cellular differentiation (Foard, 1970). Studies on sclereid formation in Camellia and root hair differentiation in wheat that grew without cell division after X-ray irradiation have led to the conclusion that unequal division is an expression of an underlying polarisation but not a determinant of cellular differentiation (Foard, 1970).

An understanding of the distinction between determination and expression of pattern formation is essential for the formulation of a universal model of plant and animal development. This distinction is clearly demonstrated by the early polar development of the Fucus zygote.

The zygotes of the brown alga, Fucus, have been used as a model system to study the development of cell polarity for almost 90 years (Quatrano, 1978). In the Fucus egg a wide variety of external gradients such as light, pH, voltage, and ions can alter the expression of polarity. Many studies have indicated that these orienting vectors may influence

polarity by redistributing particles or components of the membranes responsible for transporting calcium ions inward. There is evidence that the stabilisation of these plasma-membrane components within the membrane or to the underlying cytoplasm by microfilaments is the critical event in the determination of cell polarity in the Fucus zygote. An influx of calcium triggers the early development of the embryo of the sea urchin (Steinhardt and Epel, 1974). Thus it is conceivable that similar interactions between the plasma-membrane, calcium and microfilaments may provide the essential features in a universal model of pattern formation for plants and animals. The various components of this model are discussed in the next section 4.2.

The origin of this universality of plant and animal development may be in the evolution of multicellular organisms through the common process of chemotaxis. During chemotaxis the motile cells are enabled to respond to gradients of concentration of chemicals in their environment. The concept of positional information also involves a mechanism whereby the cells are able to detect concentration gradients. Therefore the idea that chemotaxis may be a primitive form of interpretation of positional information warrants consideration.

Carlile (1975) has discussed the possible connection between the evolution of chemotaxis and intercellular communication in morphogenesis. "Once an organism possessed a sensory system responding to chemicals, the evolution of a communication system became possible. A response to a chemical in the environment could become a response to the same chemical fortuitously emitted by another species and

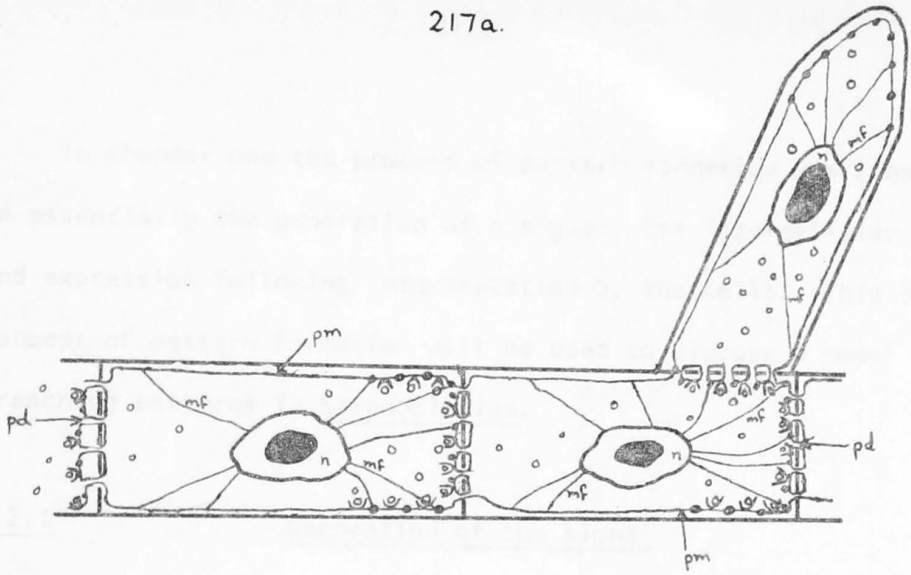
finally by cells of the same species " (Carlile, 1975). He cites the chemotaxis of Dictyostelium to cyclic AMP. The feeding of Dictyostelium amoeba depends upon their chemotactic response to cyclic AMP emitted by bacteria. This same chemotactic mechanism is used in the aggregation of amoebae. When a "founder" cell begins to emit cyclic AMP it attracts surrounding amoebae which themselves emit cyclic AMP, creating a relay system which results in aggregation. "Thus the presence of a sensory system has permitted the evolution of a communication system with a role in morphogenesis " (Carlile, 1975).

"The existence of chemical communcation between cells must have been a prerequisite for the evolution of multicellular organisms... ... Sensory systems permitted the evolution of intercellular communcation which in turn made possible the evolution of more sophisticated sensory systems of higher organisms..." (Carlile, 1975).

## 4.2 A model of pattern formation for Stigeoclonium

An understanding of pattern formation requires the analysis of the behaviour of the organism as a functional hierarchy which involves the parametric control of one level of behaviour by processes at the next (Goodwin, 1976). The levels of organisation include the electrochemical or ionic, the metabolic or biochemical, the cellular and multicellular. The value of models in developmental biology may be in the synthesis of these various levels through a delicate balance of knowledge and vision such that the model provides a bridge between the levels of biological organisation.

The studies reported here on Stigeoclonium have largely been undertaken at the cellular and multicellular level. A model is now proposed as a synthesis of these results which may act as a framework to analyse pattern formation at other levels of the functional hierarchy of biological organisation.




n , nucleus
mf , microfilament
pm , plasma-membrane
pd , plasmodesmata
• , calcium influx channels
o , morphogen
∩ , morphogen binding site

Fig. 4.1 A diagram of the major components proposed for the model of pattern formation in Stigeoclonium.

Branch initiation depends on a symplastically transported inhibitory agent ( morphogen, o ) which diffuses in a basipetal direction away from its centre of production at the branch tips. The morphogen is cyclically produced and binds to sites ( morphogen binding site, ∪ ) located in the plasma-membrane at the apical end of the cell, preventing an influx of calcium ions through the nearby calcium influx channels ( • ). When the morphogen concentration drops below a critical level and the morphogen binding sites are no longer saturated, a localised influx of calcium ions could trigger the process of branching including the movement of the nucleus by the microfilaments to the site of branch initiation. The model is discussed in more detail on pages 216 to 227.

In chapter one the process of pattern formation was considered to be essentially the generation of a signal, its intercellular transport, and expression following interpretation by the cells. This basic concept of pattern formation will be used to discuss a model of branching patterns in Stigeoclonium.

## 4.2.1 Generation of the signal

The studies presented in this thesis are held to be consistent with an interpretation of the branching patterns in Stigeoclonium as a spacing pattern maintained by an inhibitory mechanism.

The mature plant is thought to consist of a number of overlapping inhibitory fields with their source at the apical cell. The apical cell is the only differentiated cell in a young filament and it later forms a highly differentiated hair cell in older cultures. The apical cell has a morphological polarity which none of the other vegetative cells show until they initiate branches. Morphologically the branch initial and the apical cell involve areas of intensive metabolism as new membrane and cell wall are deposited at the growing tip. This tip growth is dependent on localized specific deposition of structural materials which apparently arrive at the site of incorporation as vesicles. Both membranes and cell walls in the tip region are undergoing expansion and must be in a state allowing ready incorporation of new materials.

Both the descriptive and experimental studies on Stigeoclonium would suggest that these specialised polar apical cells are the source of a branch inhibitor. The production of double branch points in

Stigeoclonium only occurs in mature cultures when the first branch tip is separated from the basal cell by a long file of cells. There is also some evidence to suggest that the apical cell inhibits rhizoid initiation from excised fragments. This could indicate as Wolpert (1969, 1971) has suggested, that similar monotonic gradients may be used to produce differentiation of various types depending on the threshold level for differentiation.

The signal could be generated at specific times during the cell cycle to provide a temporal component to pattern formation in Stigeoclonium. This temporal control is of particular importance in Stigeoclonium because of the dynamic interaction between cell division and branching in Stigeoclonium. The development of the first branch in young filaments is associated with asynchrony of division. In most cases the first branch was formed from a cell which had divided ahead of neighbouring cells in the main filament, suggesting that it reached a critical stage in its cell cycle which allowed it to be released from the effects of branch inhibition.

There is some evidence in Stigeoclonium that the cell may be more readily induced to branch during the S phase of the cell cycle. Synchronisation of cells to the later S phase with 5-aminouracil allowed a more uniform branching response to plasmolysis, suggesting that the cells were in a phase of the cell cycle which could readily be reprogrammed towards branch initiation.

## 4.2.2 Transport of the Signal

The studies on *Stigeoclonium* suggest that the intercellular transport of the branching inhibitor is symplastic *via* the plasmodesmata. For a filamentous alga such as *Stigeoclonium* which is bathed in an aqueous medium, the symplasm represents the only channel of transport which could be controlled to any degree by the plant. Apoplastic transport would result in rapid diffusion into the external medium.

The induction of branching by several techniques (plasmolysis, cutting, laser microbeam irradiation), which interfere with the continuity of symplastic transport is consistent with a model of branching controlled by the intercellular transport of a branch inhibitor through the plasmodesmata. The observed occlusion and attentuation of plasmodesmata during the formation of zoospores also suggests some role for plasmodesmata in controlling differentiation.

Some evidence was presented that for *Stigeoclonium* calcium may play a role in regulating the intercellular transport of the branch inhibitor. Callose deposition near the cross walls during plasmolysis is dependent upon free calcium and does not form in the presence of EDTA. The EDAX data suggest that there may be a build-up of calcium near the cross walls during plasmolysis.

There is considerable evidence that in animal cells calcium controls intercellular communication. Cells may communicate by the transfer of ions and/or molecules. Ionic and metabolic coupling have been extensively studied in animal cells and the communication pathway

for both forms of coupling is considered to be the gap junctions in animal cells (Gilula and Epstein, 1976).

Elegant studies by Rose and Loewenstein (1975a) have established that in Chironomus salivary gland cells the uncoupling induced by elevated levels of intracellular calcium resulted from a local effect of calcium on the gap junction. The calcium ionophore A 23187 was effective in increasing intracellular calcium levels leading to uncoupling of the Chironomus salivary gland cells (Rose and Loewenstein, 1975a). Uncoupling in Chironomus salivary gland was also observed during exposure for longer than one hour to calcium-free medium (Rose and Loewenstein, 1971). Calcium ions are rapidly sequestered by energized cellular organelles and therefore calcium spreads only over very short distances in a cell (Rose and Loewenstein, 1975b). Thus calcium uncoupling could be spatially very selective within a single cell. Rose et al. (1977) found a graded change in the junctional permeability of Chironomus salivary gland cells dependent upon the concentration of calcium. This graded control could provide a mechanism for selective transport of intercellular signals during development.

The studies on branching in Stigeoclonium during treatment with the calcium ionophore A 23187 suggest that calcium may have a role in controlling polarity of branching. The fact that prolonged ionophore treatment altered the polarity of branching suggests that calcium could be affecting the intracellular expression of branching. The role of calcium in the initiation of branching is discussed in the next section.

## 4.2.3 The interpretation and expression of the signal

This is one of the most important steps in the process of pattern formation as formulated by Wolpert (1969, 1971). The distinction between positional information and its interpretation allows a possible universal mechanism for positional information based on variant interpretations of a similar gradient of morphogen.

The studies on Stigeoclonium suggest that the interpretation and expression of the signal may require the interaction of plasma-membrane receptors with calcium ions and possibly microfilaments. Some of the known properties of these three components of the model will now be discussed.

### (i) Microfilaments

Microfilaments are involved in many important contractile functions in the cell including locomotion, cytoplasmic streaming, cell shape maintenance and control of the movement of certain plasma-membrane components (Goldman et al. 1976; Nicolson, 1976). Microfilaments contain actin (Ishikawa et al. 1969; Goldman et al. 1975) and occur as a fine network possibly connected to the plasma-membrane (Tilney and Detmers, 1975).

The process of capping in B-lymphocytes where rearrangement of ligand membrane receptors occurs, is prevented, to various degrees, by drugs which disrupt microfilaments, such as the cytochalasins (Yahara and Edelman, 1972; Poste et al. 1975 a and b), but not by drugs which

depolymerize microtubules (Unanue et al. 1973; Poste et al. 1975a). Microfilaments are considered to have an active contractile role in providing the stress needed to transport ligand-receptor clusters and patches into a cap at the end of the cell.

Durham (1974) suggested a unified theory of non-muscle movements involving actin and myosin which was controlled by calcium flows across membranes. It was assumed that calcium ions activate the actomyosin force generation process which could pull vesicles and other material towards sites of local calcium entry.

The studies on Stigeoclonium have shown that treatment with cytochalasin B alters the polarity of branch initiation. This could be due to an effect on microfilaments preventing the movement of the nucleus and associated cytoplasm or as discussed above, it could also affect the movement or localisation of membrane components at the normal polar areas of branch initiation. It is possible that these membrane components have a function in allowing a localised flux of calcium into the cell and this is discussed in the last section.

(ii) Calcium

The large difference in concentration between the intracellular calcium level (of the order of $10^{-6}$ M) and the extracellular level ($10^{-3}$ M) means that calcium would be ideal as a trigger for many cellular events since even a small increase in intracellular calcium level would be significant. The interaction between intracellular and extracellular calcium may also provide a sensitive means of interaction with the

environment for the plant. Indeed there is evidence that light may affect the permeability of the plasma-membrane to calcium. In the filamentous green alga, Mougeotia, light enhances the flux of calcium across the plasma-membrane by a factor of two (Wagner and Bellini, 1976).

Berridge (1976) has argued that calcium is a primary regulant of cell division. Mazia (1937) was one of the first to suggest that calcium might trigger cell division after fertilization. Steinhardt and Epel (1974) confirmed this by inducing with the calcium ionophore A 23187 all the initial events normally associated with fertilization in the sea urchin egg.

Studies on calcium gradients, visualized with chlorotetracyline (CTC) fluorescence, have concluded that for a number of tip-growing plant cells, the observed cytoplasmic calcium gradient may be one cause of the oriented tip growth (Reiss and Herth, 1979b). The destruction of the calcium gradient by the use of the calcium ionophore A 23187 inhibits tip growth (Herth, 1978; Reiss and Herth, 1979a). The micrographs of Reiss and Herth (1979b) for the moss caulonema of Funaria also show CTC fluorescence of the cross wall area but its possible role was not discussed.

Thus calcium may be a primary regulator of both cell division and tip growth, two of the most important processes during pattern formation in Stigeoclonium.

(iii) Interactions between calcium and the plasma-membrane

A renaissance in research on cell membranes has occurred since the publication of the "Fluid Mosaic Model" of cell membranes (Singer and Nicolson, 1972). Membranes were visualized as phospholipid bilayers in a "fluid" dynamic state with numerous proteins inserted into the lipid bilayer. Calcium plays a possible regulatory role in the modulation of properties of biological membranes. Manery (1966) concluded that many of the effects of calcium ions on membranes could be attributed to the ease with which they formed very stable chelates. Calcium condenses phospholipid bilayers and restricts the mobility of anionic phospholipids as revealed by spin-labelled stearic acid analogues (Schnepel et al. 1974). Calcium binding also results in decreased mobility of ESR probes in Bacillus subtilis (Ehrström et al. 1973) and rat liver plasma-membranes (Sauerheber and Gordon, 1975). There is increasing evidence that $Ca^{2+}$ influx into cells may serve as an important initial transmembrane signal for triggering the response of cells to hormones, transmitters and possibly mitogens (Rasmussen, 1970; Poste and Allison, 1973; Douglas, 1974; Carafoli et al. 1975). Changes in calcium binding to membranes can be triggered by a number of biologically active molecules including cyclic nucleotides, ATP, neurotransmitters and some hormones (Triggle, 1972; Rubin, 1974; Berridge, 1975; Carafoli et al. 1975). These changes in calcium binding to membranes could produce structural re-organisations such as topographic rearrangement of membrane components and changes in membrane properties.

It has been suggested that areas of membrane stress and deformation may allow localized calcium influx. Naitoh and Eckert (1969) found

that when Paramecium encountered an obstacle, membrane deformation opened calcium channels at the anterior end of the animal and the subsequent calcium influx triggered ciliary reversal. Nuccitelli and Jaffe (1976) have also suggested that the growing rhizoid tip of the brown alga, Pelvetia, may be a site for calcium entry due to the opening of channels by the streching of the membrane. This would be consistent with the CTC-fluorescence of tip growing cells (Reiss and Herth, 1979b).

Similar processes may explain much of the development of Stigeoclonium. The inhibitor of branching could bind to sites in the plasma-membrane which regulate calcium influx. These sites would be distributed non-randomly in the plasma-membrane with a greater concentration of sites at the apical end. The distribution of the sites could be stabilised by the microfilaments which could also aid in the movement of the nucleus. The branching of Stigeoclonium could be affected by both changes in intercellular communication of the inhibitor (plasmolysis, laser shots, cutting etc.) as well as by artificially induced influxes of calcium (ionophore). Some of the effects of plasmolysis could be due to a stretching of the plasma-membrane near the cross walls. This is the area which retracts first during plasmolysis. An influx of calcium could result but would be greatest at the apical end where it is hypothesized that the plasma-membrane sites regulating calcium influx are concentrated. The loss of polarity during prolonged plasmolysis could be explained by a disturbance in the distribution of these plasma-membrane sites as discussed at the end of Chapter 3.

The model presented in this final chapter provides a perspective and framework for future studies on pattern formation and intercellular communication in Stigeoclonium. The expression of branches in Stigeoclonium involves a dynamic interaction between the nucleus, the cytoplasm and the plasma-membrane which may be determined by the intercellular communication of an inhibitor to branching via the plasmodesmata.

The studies in this thesis have established the importance of symplastic continuity maintained by the plasmodesmata for the regulation of branching patterns in Stigeoclonium. The nature of this ionic and chemical coupling between the cells of a filament still remains to be established. The size of molecules which can pass through plasmodesmata and the degree of ionic coupling between cells could be studied using electrophysiological techniques for procion dye injection and current injection to test ionic coupling. Preliminary attempts at these studies indicate that the small size of Stigeoclonium cells may make the application of these techniques difficult.

The experimental studies on Stigeoclonium have clearly demonstrated the importance of calcium for both the position and polarity of branches. It would be worthwhile to investigate the effect of intracellular calcium levels on the degree of ionic and chemical coupling between cells. In particular since it has been established in Chapter 3 that free calcium is necessary for callose formation it would be of considerable interest to establish the permeability of callose plugs in the plasmodesmata to both ions and molecules. Further EDAX studies may be useful to determine if there is an intracellular gradient of calcium associated with the cell

cortex while CTC-fluorescence could be used to possibly detect the site of branch initiation during an influx of calcium ions.

The disruption of branch polarity by cytochalasin B treatment suggested that microfilaments may be involved in the expression of polar branch initiation. The occurrence and distribution of microfilaments in Stigeoclonium cells could be established using immunofluorescent techniques. In particular the studies in this thesis suggest it would be of interest to establish the association of microfilaments with the plasma-membrane and the nucleus.

Freeze-etch studies of the plasma-membrane of Stigeoclonium during branch initiation may be of special significance. The studies presented in this thesis suggest that the effect of cytochalasin B on both microfilaments and the distribution of intra-membranous particles could provide insights into the role of the plasma-membrane in controlling polarity of branching in Stigeoclonium.

## BIBLIOGRAPHY

ABBAS, A. and GODWARD, M.B.E. 1963. Effects of experimental culture in Stigeoclonium.
Br. Phycol. Bull. 2: 281-282.

ADLER, I. 1974. A model of contact pressure in phyllotaxis.
J. Theor. Biol. 45: 1-79.

AIST, J.R. 1976. Papillae and related wound plugs of plant cells.
Ann. Rev. Phytopathol. 14: 145-63

AKSAMIT, R. and KOSHLAND, E. Jnr. 1972. A ribose binding protein of Salmonella typhimurium.
Biochem. Biophys. Res. Commun. 48: 1348-1353.

ALLEN, R. 1974. Some new insights concerning cytoplasmic transport.
Soc. Exp. Biol. Symposium 28: 15-26

AMAR, L. and REINHOLD, L. 1973. Loss of membrane transport ability in leaf cells and release of protein as a result of osmotic shock.
Plant Physiol. 51: 620-5.

ANRAKU, Y. 1968. Transport of sugars and amino acids in bacteria. I. Purification and specificity of the galactose- and leucine-binding protein.
J. Biol. Chem. 243: 3116-22.

ASPINALL, G.O. and KESSLER, G. 1957. The structure of callose from the grape vine.
Chem. and Ind. (London): 1296.

ATLAN, H. 1975. Source and transmission of information in biological networks. From: Miller, I.R. (Ed.).
Stability and Origin of Biological Information. p.95-118.
John Wiley and Sons, New York.

BABLOYANTZ, A. and HIERNAUX, J. 1975. Models for cell differentiation and generation of polarity in diffusion governed morphogenetic fields.
Bull. Math. Biol. 37: 637-657

BAKER, P.F. 1976. The regulation of intracellular calcium.
Soc. Exp. Biol. Symposium 30: 67-88

BALL, E. 1948. Differentiation in the primary shoots of Lupinus albus L. and of Tropaeolum majus L.
Soc. Exp. Biol. Symposium 2: 246-62.

BARDSETH, E. 1965. Localization and structure of alginate gels. In : Young, E.G. and McLachlan, J.L. (Eds.). Proc. 5th Int. Seaweed Symposium p. 19-28 Pergamon Press.

BARLOW, P.W. 1976. Towards an understanding of the behaviour of root meristems. J. Theor. Biol. 57: 433-51.

BAUER, L. 1957. Regenerationsversuche am Sporogon von Physcomitrium piriforme (Brid.). Ber. dtsch. bot. Ges. 70: 424-32.

BECERRA, J. and LÓPEZ-SÁEZ, J.F. 1978. Effects of caffeine, calcium and magnesium on plant cytokinesis. Exp. Cell Research 111: 301-8.

BERGER, E.A. and HEPPEL, L.A. 1972. A binding protein involved in the transport of cystine and diaminopimelic acid Escherichia coli. J. Biol. Chem. 247: 7684-94.

BERKING, S. 1977. Bud formation in Hydra - inhibition by an endogeneous morphogen. W.Roux's Archive, 181: 215-225.

BERLINER, M.D. 1971. Induction of protoplasts of Schizosaccharomyces octosporus by magnesium sulfate and 2-deoxy-d-glucose. Mycologia63: 819-23.

BERLINER, M.D. and RECA, M.E. 1970. Release of protoplasts in the yeast phase of Histoplasma capsulatum without added enzyme. Science 167: 1255-7.

BERLINER, M.D. and WENC, K.A. 1976. Osmotic pressure effects and protoplast formation in Cosmarium turpinii. Microbios Letters 2: 39-45.

BERRIDGE, M.J. 1975. The interaction of cyclic nucleotides and calcium in the control of cellular activity. Advances in Cyclic Nucleotide Research 6: 1-98.

BERRIDGE, M.J. 1976. Calcium, cyclic nucleotides and cell division. Soc. Exp. Biol. Symposium 30: 219-31.

BIRGIT, R., SIMPSON, I., and LOEWENSTEIN, W.R. 1977. Calcium ion produces graded changes in permeability of membrane channels in cell junction. Nature 267: 625-7.

BLOCH, R. 1965a. Histological foundations of differentation and development in plants.
In: Handbuch der Pflanzenphysiologie XV/1: 146-88. Springer-Verlag, Berlin.

BLOCH, R. 1965b. Polarity and gradients in plants: A survey.
In: Handbuch der Pflanzenphysiologie XV/1: 234-74. Springer-Verlag, Berlin.

BLUEMINK, J.G. and TERTOOLEN, L.G.J. 1978. The plasma-membrane IMP pattern as related to animal/vegetal polarity in the amphibian egg.
Dev. Biol. 62: 334-43.

BONNETT, H.T. Jnr. and NEWCOMB, E.H. 1966. Coated vesicles and other cytoplasmic components of growing root hairs of radish.
Protoplasma 62: 59-75.

BORISY, G.G. and TAYLOR, E.W. 1967. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus.
J.Cell Biol. 34: 535-48.

BORLE, A.B. and ANDERSON, J.H. 1976. A cybernetic view of cell calcium metabolism.
Soc. Exp. Biol. Symposium 30: 141-160.

BRACHET, J. 1968. Synthesis of macromolecules and morphogenesis in Acetabularia.
Curr. Top. Dev. Biol. 3: 1-36.

BRITTEN, R.J. and DAVIDSON, E.H. 1969. Gene regulation for higher cells: a theory.
Science 165: 349-357.

BRUNNER, G. 1977. Membrane impression and gene expression.
Differentiation 8: 123-132.

BRUNORI, A. 1967. Relationship between DNA synthesis and water content during ripening of Vicia faba seeds.
Caryologia 20: 333-8.

BÜNNING, E. 1948. Entwicklung und Bewegungsphysiologie der Pflanze.
Springer-Verlag, Berlin.

BÜNNING, E. 1952. Morphogenesis in plants.
Survey of Biological Progress 2: 105-40.

BÜNNING, E. 1965. Die Entstehung von Mustern in der Entwicklung von Pflanzen.
In: Tuhland, W. (Ed.)
Encyclopedia of plant physiology. Vol.15. pt.1. p.383-408.
Springer-Verlag, Berlin.

BÜNNING, E. and SAGROMSKY, H. 1948. Die Bildung des Spaltöffnungsmusters in der Blattepidermis.
Z. Naturforsch. 3b: 203-16.

BURGESS, J. 1971. Observations on structure and differentiation in plasmodesmata.
Protoplasma 73: 83-95.

BUTCHER, R.W. 1950. Some experiences in the culture of algae.
Int.Assoc.Theoret.Appl.Limnol. 11: 66-68.

BYGRAVE, F.I. 1978. Mitochondria and the control of intracellular calcium.
Biol. Rev. 53: 43-79.

CAMPBELL, R.D. 1979. Development of Hydra lacking intestinal and nerve cells ("Epithelial Hydra").
Symp. Soc. Dev. Biol. 37: 267-93.

CARAFOLI, E. 1974. Mitochondrial uptake of calcium ions and the regulation of cell function.
Biochem. Soc. Symp. 39: 89-109.

CARAFOLI, E. and CROMPTON, M. 1976. Calcium ions and mitochondria.
Soc.Exp.Biol.Symposium 30: 89-115.

CARAFOLI, E., CLEMENTI, F., DRABIKOWSKI, W., and MARGRETH, A. 1975.(Eds.) Calcium Transport in Contraction and Secretion, North-Holland, Amsterdam.

CARDE, J.P. 1974. Le Tissu de transfert (= cellulues de Strasburger) dans les aiguilles du pin maritime (Pinus pinaster Ait.). II. Caractères cytochimiques et infrastructuraux de la pario et des plasmodesmes.
J. Microscopie 20: 51-72.

CARLILE, M.J. 1975. Taxes and tropisms: Diversity, biological significance and evolution from Carlile, M.J. (Ed.).
Primitive Sensory and Communication Systems : 1-28, Academic Press.

CARR, D.J. 1976. Plasmodesmata in growth and development. In: Gunning, B.E.S. and Robards, A.W. (Eds),
Intercellular Communication in Plants: Studies on Plasmodesmats.
p. 243-87, Springer-Verlag, Heidelberg.

CARTER, S. 1972. The cytochalasins as research tools in cytology.
Endeavour 31: 77-82.

CERCEK, L., CERCEK, B., and OCKEY, C.H. 1973. Cell membrane permeability during the cell generation cycle in Chinese hamster ovary cells.
Biophysik 10: 195-7.

*
CHILD, C.M. 1915. Individuality in Organisms.
Chicago University Press, Chicago.

*CHANG, J.P. 1971. A new technique for separation of coverglass substrate from epoxy-embedded specimens for electron microscopy.
J. Ultrastruct. Res. 37 : 370-3.

CHILD, C.M. 1941. Patterns and Problems of Development.
Chicago University Press, Chicago.

CONKLIN, E.G. 1917. Effects of centrifugal force on the structure and development of the eggs of Crepidula.
J. Exp. Zool. 22: 311-420.

COOKE, J. 1975. The emergence and regulation of spatial organisation in early animal development.
Ann. Rev. Biophys. Bioeng. 4: 185-217.

COX, E.R. and BOLD, H.C. 1966.
Phycological Studies VII Taxonomic investigations of Stigeoclonium.
University of Texas Publication No.6612. 167 pp.

CRESTI, M. and VAN WENT, J.L. 1976. Callose deposition and plug formation in Petunia pollen tubes in situ.
Planta 133: 35-40.

CRICK, F. 1970. Diffusion in embryogenesis.
Nature 225: 420-422.

CRICK, F.H.C. 1971. The scale of pattern formation.
Soc. Exp. Biol. Symposium. 25: 429-438.

CURRIER, H.B. 1957. Callose substance in plant cells.
Amer. J. Bot. 44: 478-88.

CURRIER, H.B. and STRUGGER, S. 1956. Aniline blue and fluorescence microscopy of callose in bulb scales of Allium cepa L.
Protoplasma 45: 552-9.

CURRIER, H.B. and WEBSTER, D.H. 1964. Callose formation and subsequent disappearance: studies in ultrasound stimulation.
Plant Physiol. 39: 843-48.

CZAJA, A.T. 1930. Zellphysiologische Untersuchungen an Cladophora glomerata. Isolierung, Regeneration und Polarität.
Protoplasma II: 601-27.

DALCQ, A.M. 1938. Form and Causality in Early Development.
Cambridge University Press, Cambridge.

DALCQ, A., and PASTEELS, J. 1937. Une conception nouvelle des bases physiologiques de la morphogenese.
Arch. de Biol. 48: 669-710.

DALCQ, A., and PASTEELS, J. 1938. Potentiel morphogenetique, regulation et "Axial Gradients" de Child. Mise au Point des "Bases physiologiques de la morphogenese".
Bull. Acad. Roy. Med. Belg. 3: 261-308.

*1. DODDS, J.H. 1979. Is cell cycle activity necessary for xylem cell differentiation? What's New in Plant Physiology 10: 13-16.

D'AMATO, F. 1972. Morphogenetic aspects of the development of meristems in seed embryos. In: Miller, M.W., and Keuhnert, C.C. (Eds.) The Dynamics of meristem cell populations p. 149-63. Plenum Pub. Corp., N.Y.

DE KAZOS, E.D., and WORLEY, J.F. 1967. Induction of callose formation by bruising and aging of red tart cherries. J. Food Sci. 32: 287-89.

DIÉZ, J.L., GONZÁLEX-FERNÁNDEZ, A., and LÓPEZ-SÁEZ, J.F. 1976. Mechanism of mitotic synchronization induced by 5-aminouracil. Exp. Cell Research 98: 79-89.

*2. DOUGLAS, W.W. 1974. Mechanism of release of neurophypophysial hormones: stimulus secretion-coupling. In: Handbook of Physiology, Vol. 1V, Section 7, American Physiological Society, Washington, D.C. p. 191-224.

DRAKE, G.A., CARR, D.J. and ANDERSON, W.P. 1978. Plasmolysis, plasmodesmata and the electrical coupling of oat coleoptile cells. J. Exp. Bot. 29: 1205-14.

DRIESCH, H. 1908. Science and Philosophy of the Organism. A. and C. Black, London.

DUFF, M.J.B. 1977. Pattern recognition. Sci. Prog., Oxf. 64: 423-445.

DURHAM, A.C.H. 1974. A unified theory of the control of actin and myosin in non-muscle movements. Cell. Vol 2: 123-35.

EHRSTROM, M., ERIKSSON, L.E.G., ISRAELACHVILI, J., and EHRENBERG, A. 1973. The effects of some cations and anions on spin labeled cytoplasmic membranes of Bacillus subtilis. Biochem. Biophys. Res. Commun. 55: 396-402.

ENNIS, H.L. and LUBIN, M. 1964. Cycloheximide : Aspects of inhibition of protein synthesis in mammalian cells. Science 146: 1474-6.

ESCHRICH, W. 1956. Kallose (Ein kritischer Sammelbericht.) Protoplasma 47: 487-530.

ESCHRICH, W. 1957. Kallosebildung in plasmolysiert Allium cepa-Epidermen. Planta 48: 578-86.

ESCHRICH, W. 1965. Physiologie der Siebröhrencallose. Planta 65: 280-300.

ESCHRICH, W., and CURRIER, H.B. 1964. Identification of callose by its diachrome and fluorochrome reactions. Stain Technol. 39: 303-7.

*2 DOOLEY, L. B. and MARCHANT, H. J. 1977. Branching patterns in the filamentous alga Stigeoclonium. Proc. VIIIth International Congress of Developmental Biologists p.82.

ESCHRICH, W., HUTTERMAN, A., HEYSER, W., TAMMES, P.M.L., and VAN DIE, J. 1972. Evidence for the synthesis of "callose" in sieve-tube exudate of Yucca flaccida.
Z. Pflanzenphysiol. 67: 468-70.

EVERT, R.F. and DERR, W.F. 1964. Callose substance in sieve elements.
Am. J. Bot. 51: 552-9.

FIFE, P.C. 1977. On modelling pattern formation by activator inhibitor systems.
J. Math. Biol. 4: 353-362.

FLEMING, H. and HASELKORN, R. 1973. Differentiation in Nostoc muscorum: nitrogenase is synthesised in heterocysts.
Proc.Natl.Acad.Sci. U.S.A. 70: 2727-31.

FLOYD, G.L., STEWART, K.D., and MATTOX, K.R. 1972. Comparative cytology of Ulothrix and Stigeoclonium.
J. Phycol. 8: 68-81.

FOARD, D.E. 1970. Differentiation in plant cells. In: Schjeide, O.A. and De Vellis, J. (Eds.).
Cell Differentiation p. 575-602.
Van Nostrand, Reinhold Co., N.Y.

FOGG, G.E. 1949. Growth and heterocyst production in Anabaena cylindrica. II. In relation to carbon and nitrogen metabolism.
Ann. Bot. N.S. 13: 241-59.

FRASER, T.W. and GUNNING, B.E.S. 1969. The ultrastructure of plasmodesmata in the filamentous green alga, Bulbochaete hiloensis (Nordst.) Tiffany.
Planta 88: 244-54.

FRASER, T.W. and GUNNING, B.E.S. 1973. Ultrastructure of the hairs of the filamentous green alga Bulbochaete hiloensis (Nordst.) Tiffany: an apoplastidic plant cell with a well developed Golgi apparatus.
Planta 113: 1-19.

FREDERICK, S.E., GRUBER, P.J. and TOLBERT, N.E. 1973. The occurrence of glycolate dehydrogenase and glycolate oxidase in green plants. An evolutionary survey.
Pl. Physiol. 52: 318-23.

FRITSCH, F.E. 1935. Structure and reproduction of the algae Vol.I.
Cambridge University Press

FRITSCH, F.E. 1951. The heterocyst: a botanical enigma.
Proc. Linn. Soc. London 162: 194-211.

FULCHER, R.G., McCULLY, M.E., SETTERFIELD, G., and SUTHERLAND, J. 1976. β,1-3 Glucans may be associated with cell plate formation during cytokinesis.
Can. J. Bot. 54: 539-42.

GARCÍA-ARENAL, F. and SAGASTA, E.M. 1977. Callose deposition and phytoalexin accumulation in Potrytis cinerea-infected bean (Phaseolus vulgaris).
Plant Science Letters 10: 305-12.

GASKING, E. 1970. The Rise of Experimental Biology.
Random House, N.Y.

GAWLIK, S.R. and MILLINGTON, W.F. 1969. Pattern formation and the fine structure of the developing cell wall in colonies of Pediastrum boryanum.
Am. J. Bot. 56: 1084-93.

GIERER, A., and MEINHARDT, H. 1972. A theory of biological pattern formation.
Kybernetik. 12: 30-39.

GILLBANK, L.R., ROWAN, K.S., and SPRING, A. 1972. The after-response of growth and respiration of sections of wheat coleoptile following withdrawal of cyanide.
Cytobios. 6: 119-31.

GOLDBERG, I.H. and RABINOWITZ, M. 1962. Actinomycin D inhibition of deoxyribonucleic acid-dependent synthesis of ribonucleic acid.
Science136: 315-6.

GILULA, N.B. and EPSTEIN, M.L. 1976. Cell-to-cell communication, gap junctions and calcium.
Soc. Exp. Biol. Symposium 30: 257-72.

GODWARD, M.B.E. 1942. The life cycle of Stigeoclonium amoneunum Kütz.
New Phytol. 41: 293-301.

GOLDMAN, R.D., LAZARIDES, E., POLLACK, R., and WEBER, K. 1975.
The distribution of actin in non-muscle cells. The use of actin antibody in the localization of actin within the microfilament bundles of mouse 3T3 cells.
Exp. Cell Res. 90: 333-344.

GOLDMAN, R., POLLARD, T., ROSENABUAM,T.&J., (Eds.) 1976.
Cell Motility, Cold Spring Harbour Conferences on Cell Proliferation. Vol.3., Cold Spring Harbour, N.Y.

GOODWIN, B.C. 1976. Analytical physiology of cells and developing organisms.
Academic Press, London.

GOODWIN, B.C., and COHEN, M.H. 1969. A phase-shift model for the spatial and temporal organisation of developing systems.
J. Theor. Biol. 25: 49-107.

GOODWIN, B.C., and PATEROMICHELAKIS, S. 1979. The role of electrical fields, ions, and the cortex in the morphogenesis of Acetabularia.
Planta 145: 427-35.

GRAHAM, C.F., and WAREING, P.F. 1976.
The Developmental Biology of Plants and Animals
Blackwell, Oxford.

GREEN, P.B. 1968. Growth physics in Nitella: a method for continuous in vivo analysis of extensibility, based on a micro-manometer technique for turgor pressure.
Plant Physiol. 43: 1169-84.

GROVE, S.N., and BRACKER, C.E. 1970. Protoplasmic organization of hyphal tips among fungi: vesicles and spitzenkörper.
J. Bacteriol. 104: 989-1009.

GUNNING, B.E.S., and ROBARDS, A.W. 1976.
Intercellular Communication in Plants: Studies on Plasmodesmata.
Springer-Verlag, Berlin.

GUNNING, B.E.S., HUGHES, J.E., and HARDHAM, A.R. 1978a. Formative and proliferative cell divisions, cell differentiation and developmental changes in the meristem of Azolla roots.
Planta 143: 121-144.

GUNNING, B.E.S., HARDHAM, A.R., and HUGHES, J.E. 1978b. Pre-prophase bands of microtubules in all categories of formative and proliferative cell division in Azolla roots.
Planta 143: 145-160.

HÄMMERLING, J. 1963. Nucleo-cytoplasmic interactions in Acetabularia and other cells.
Ann. Rev. Plant Physiol. 14: 65-92.

HART, J.W., and SABNIS, D.D. 1976. Colchicine and plant microtubules: A critical evaluation.
Current Advances in Plant Sciences 26: 1095-1104.

HAUPT, W. 1972. Perception of light direction in oriented displacements of cell organelles.
Acta Protozoologica 11: 179-88.

HAUPT, W., and WEISENSEEL, M.H. 1976. Physiological evidence and some thoughts on localised responses, intracellular localisation and action of phytochorme. In: Smith, H. (Ed.)
Light and plant development. p. 63-74. Butterworths, London.

HAY, E.D. 1966. Regeneration.
Holt, Rinehart and Winston. New York.

HAZELBAUER, G.L. and ADLER, J. 1971. Role of the galactose binding protein in chemotaxis of Escherichia coli toward galactose.
Nature New Biol. 230: 101-4.

HELSPER, J.P.F.G., VEERKAMP, J.H., and SASSEN, M.M.A. 1977. β-Glucan synthetase activity in golgi vesicles of Petunia hybrida.
Planta 133: 303-8.

HEPLER, P.R., and PALEVITZ, B.A. 1974. Microtubules and microfilaments.
Ann. Rev. Plant Physiol. 25: 309-62.

HEPPEL, L.A. 1967. Selective release of enzymes from bacteria.
Science 156: 1451-5.

HERTH, W. 1978. Ionophore A 23187 stops tip growth, but not cytoplasmic streaming, in pollen tubes of Lilium longiflorum.
Protoplasma 96: 275-82.

HINZ, F.A. 1974. Untersuchungen über Wachstum und Vermehrung von Grunalgen unter Coffein ein fluss.
Arch. Protistenk. 116: 295-303.

HOLDER, N. 1979. Positional information and pattern formation in plant morphogenesis and a mechanism for the involvement of plant hormones.
J. Theor. Biol. 77: 195-212.

HOLTZER, H. 1963. Mitosis and cell transformation. In (Eds. Mazia, D., and Tyler, A).
General Physiology of Cell Specialization. p.80-112
McGraw-Hill, N.Y.

HSIAO, T.C., ACEVEDO, E., and HENDERSON, D.W. 1970. Maize-leaf elongation: continuous measurements and close dependence on plant water status.
Science 168: 590-1.

HUGHES, J., and McCULLY, M.E. 1975. The use of an optical brightener in the study of plant structure.
Stain technology 50: 319-29.

IGURA, I. 1955. Cytological and morphological studies on the gametophytes of ferns IX. The polar plasmolysis of fern-prothallium (1), (2), (3), (4).
Bot. Mag. Tokyo 68: 119-24, 184-6, 208-12, 289-93.

ILKER, R., WARING, A.J., LYONS, J.M., and BREIDENBACH, R.W. 1976. The cytological responses of tomato-seedling cotyledons to chilling and the influence of membrane modifications upon these responses. Protoplasma 90: 229-252.

ISHIKAWA, H., BISCHOFF, R., and HOLTZER, H. 1969. Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. J. Cell Biol. 43: 312-28.

ISLAM, A.K.M. 1963. Revision of the genus Stigeoclonium. Nova Hedwig, 10 J. Cramer, Weinheim 168 pp.

ITO, M. 1962. Studies on the differentiation of fern gametophytes 1. Regeneration of single cells isolated from cordate gametophytes of Pteris vittata. Bot. Mag. Tokyo 75: 19-27.

JACOB, F., and MONOD, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

JACOBS, W.P. 1952. The role of auxin in differentiation of xylem around a wound. Am.J.Bot. 39: 301-9.

JAFFE, L.F., and NUCCITELLI, R. 1977. Electrical controls of development. Ann. Rev. Biophys. Bioeng. 6: 445-76.

JAFFE, L., ROBINSON, K.R., NUCCITELLI, R. 1974. Local cation entry and self-electrophoresis as an intracellular localization mechanism. Ann. N.Y. Acad. Sci. 238: 372-89.

JEFFS, R.A., and NORTHCOTE, D.H. 1967. The influence of indol 3-yl acetic acid and sugar on the pattern of induced differentiation in plant tissue culture. J.Cell Sci. 2: 77-88.

KALCKAR, H.M. 1971. The periplasmic galactose binding protein of Escherichia coli. Science 174: 557-68.

KAUFFMAN, S.A. 1969. Metabolic stability and epigenesis in randomly constructed genetic maps. J.Theor. Biol. 22: 437-467.

KAUFFMAN, S.A., SHYMKO, R.M., and TRABERT, K. 1978. Control of sequential compartment formation in Drosophila. Science 199: 259-270.

KESSEL, R.G. 1973. Structure and function of the nuclear envelope and related cytomembranes.
Prog.Surf. and Memb.Sci. 6: 243-329.

KESSLER, G. 1958. Zur Charackterisierung der Siebröhrenkallose.
Ber. Schweiz. Bot. Ges. 68: 5-43.

KISHIMOTO, S., and LIEBERMAN, I. 1964. Synthesis of RNA and protein required for the mitosis of mammalian cells.
Exp. Cell Res. 36: 92-101.

KLEBS, G. 1896. Die Bedingungen der Fortpflanzung bei einiger Algen und Pilzen.
Jena 543 pp.

KLETZIEN, R., and PERDUE, J. 1973. The inhibition of sugar transport in chick embryo fibroblasts by cytochalasin B. Evidence for a membrane-specific effect.
J. Biol. Chem. 248: 711-9.

KNOX, R.B., and HESLOP-HARRISON, J. 1970. Direct demonstration of the low permeability of the angiosperm meiotic tetrad using a fluorogenic ester.
Z. Pflanzenphysiol. 62: 451-9.

KWIATKOWSKA, M., and MASZEWSKI, J. 1976. Plasmodesmata between synchronously and asynchronously developing cells of the antheridial filaments of Chara vulgaris L.
Protoplasma 87: 317-27.

LACALLI, T.C., and HARRISON, L.G. 1978. The regulatory capacity of Turing's model for morphogenesis, with application to slime moulds.
J. Theor. Biol. 70: 273-295.

LANG, N.J. and FAY, P. 1971. The heterocysts of blue-green algae. II. Details of ultrastructure.
Proc. R. Soc. London Ser. B. 178: 193-203.

LAWRENCE, P.A. 1966a. Gradients in the insect segment: the orientation of hairs in the milkweed bug, Oncopeltus fasciatus.
J. Exp. Biol. 44: 607-20.

LAWRENCE, P.A. 1966b. Development and determination of hairs and bristles in the milkweed bug, Oncopeltus fasciatus (Lygaeidae, Hemiptera).
J. Cell Sci. 1: 475-98.

LAWRENCE, P.A. 1966c. The hormonal control of the development of hairs and bristles in the milkweed bug, Oncopeltus fasciatus, Dall.
J. Exp. Biol. 44: 507-22.

LAWRENCE, P.A. 1969. Cellular differentiation and pattern formation during metamorphosis of the milkweed bug Oncopeltus.
Dev. Biol. 19: 12-40.

LAWRENCE, P.A. 1970. Polarity and patterns in the postembryonic development of insects.
Adv. Insect Physiol. 7: 197-260.

LEAVER, C.L., and LOVETT, J.S. 1974. An analysis of protein and RNA synthesis during encystment and outgrowth (germination) of Blastocladiella zoospores.
Cell Differentiation 3: 165-92.

LICHTENSTEIN, A.V., and SHAPOT, V.S. 1976. A model for cytoplasm-governed gene regulation.
Biochem. J. 159: 783-94.

LIN, S., and SPUDICH, J. 1974a. Binding of cytochalasin B to a red cell membrane protein.
Biochem. Biophys. Res. Commun. 6: 1471-6.

LIN, S., and SPUDICH, J. 1974b. Biochemical studies on the mode of action of cytochalasin B. Cytochalasin B binding to red cell membrane in relation to glucose transport.
J. Biol. Chem. 249: 5778-83.

LIN, S., and SPUDICH, J. 1974c. On the molecular basis of action of cytochalasin B.
J. Supramolec. Struct. 2: 728-36.

LIN, S., SANTI, D., and SPUDICH, J. 1974. Biochemical studies on the mode of action of cytochalasin B. Preparation of [$^{3}$H] cytochalasin B and studies on its binding to cells.
J. Biol. Chem. 249: 2268-74.

LOEWENSTEIN, W.R. 1968. Communication through cell junctions. Implications in growth control and differentiation.
Dev. Biol. Suppl. 2: 151-83.

LÓPEZ-SÁEZ, J.F., RISUENO, M.C., and GIMÉNEZ-MARTÍN, G. 1966. Inhibition of cytokinesis in plant cells.
J. Ultrastruct. Res. 14: 85-94.

MANERY, J.F. 1966. Effects of Ca ions on membranes.
Fedn. Proc. 25: 1804-10.

MANTON, I. 1964. Observations on the fine structure of the zoospore and young germling of Stigeoclonium.
J. Exp. Bot. 15: 399-411.

MARCHANT, H.J. 1973. Processing small delicate biological specimens for scanning electron microscopy.
J. Microscopy 97: 369-71.

MARCHANT, H.J. 1976. Plasmodesmata in algae and fungi. In: Gunning, B.E.S., and Robards, A.W. (Eds.)
Intercellular Communication in Plants: Studies on Plasmodesmata. p. 58-80 Springer-Verlag, Berlin.

MARCHANT, H.J. 1978. A simple laser microbeam system for cell irradiation.
J. Microscopy 113: 311-4.

MARCHANT, H.J., and FOWKE, L.C. 1977. Preparation, culture, and regeneration of protoplasts from filamentous green algae.
Can. J. Bot. 55: 3080-6.

MARCHANT, H.J., and PICKETT-HEAPS, J.D. 1972. Ultrastructure and differentiation of Hydrodictyon reticulatum 111. Formation of vegetative daughter net.
Aust. J. Biol. Sci. 25: 265-78.

MARCHANT, H.J., PICKETT-HEAPS, J.D., and JACOBS, K. 1973. An ultrastructural study of zoosporogenesis and the mature zoospore of Klebsormidium flaccidium.
Cytobios 8: 95-107.

MARCUM, B.A., and CAMPBELL, R.D. 1978. Development of hydra lacking nerve and intestitial cells.
J. Cell Sci. 29: 17-33.

MARTINEZ, H.M. 1972. Morphogenesis and chemical dissipative structures: a computer simulated case study.
J. Theor. Biol. 36: 479-501.

MARX, A., and SACHS, T. 1977. The determination of stomata pattern and frequency in Anagallis.
Bot. Gaz. 138: 385-92.

MATILE, P., and WIEMKEN, A. 1976. Interaction between cytoplasm and vacuole. In: Stocking, C.R. and V. Heber (Eds.)
Transport in Plants 111 p. 255-87.
Springer-Verlag, Berlin.

MAZIA, D. 1937. The release of calcium in Arbacia eggs on fertilization.
J. Cell comp. Physiol. 10: 291-304.

MEINHARDT, H. 1978. Models for the ontogenetic development of higher organisms.
Rev. Physiol. Biochem. Pharmacol. 80: 47-104.

MEINS, F. Jr. 1975. Cell division and the determination phase of cyto-differentiation in plants. In: Reinhart, J., and Holtzer, H. (Eds.)
Cell Cycle and Cell Differentiation p. 151-75. Springer-Verlag, Berlin.

MELCHERS, G. 1965. Einige genetische Gesichtspunkte zu sogenannten Gwebekulturen.
Ber dtsch. bot. Ges. 78: 21-9.

MICHALKE, W. 1977. A gradient of diffusible substance in a monolayer of cultured cells.
J. Memb. Biol. 33: 1-20.

MIEHE, H. 1901. Über Wanderungen des pflanzlichen Zellkernes.
Flora 88: 105-42.

MIRANDA, A.F., GODMAN, G.C., DEITCH, A.D., and TANENBAUM, S.W. 1974. Action of cytochalasin D on cells of established lines. I. Early events.
J. Cell Biol. 61: 481-500.

MITCHISON, G.J. and WILCOX, M. 1973. Alteration in heterocyst pattern of Anabaena produced by 7-azatryptophan.
Nature New Biol. 246: 229-33.

MITCHISON, G.J. and WILCOX, M. 1972. Rule governing cell division in Anabaena.
Nature 239: 110-1.

MIZEL, S., and WILSON, L. 1972. Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B.
J. Biol. Chem. 247: 4102-5.

MOLLENHAUER, H.H., and MORRÉ, D.J. 1976. Cytochalasin B, but not colchicine, inhibits migration of secretory vesicles in root tips of maize.
Protoplasma 87: 39-48.

MORGAN, T.H. 1901. Regeneration.
Macmillan, New York.

MORRÉ, D.J., and VAN DER WOUDE, W.J. 1974. Origin and growth of cell surface components.
Symp. Soc. Dev. Biol. 30: 81-111.

MOSEBACH, G. 1943. Die Polarisierung der Equisetum - spore durch Licht.
Planta 33: 340-387.

MURRAY, J.D. 1976. On travelling wave solutions in a model for the Belousov-Zhabotinskii reaction.
J. Theor. Biol. 56: 329-353.

McLEAN, R.O., and BENSON-EVANS, K. 1977. Water chemistry and growth form variations in Stigeoclonium tenue Kutz.
Br. Phycol. J. 12: 83-88.

NAGAI, I. 1914. Physiologische Untersuchungen über Farnprothallien.
Flora 6: 281-330.

NAGAI, I. 1915. On the influence of nutrition upon the development of sexual organs in the fern prothallia.
Jour. Coll.Agric. Univ. Tokyo. 6: 112-64.

NAITOH, Y., and ECKERT, R. 1969. Ionic mechanisms controlling behavioral responses of Paramecium to mechanical stimulation. Science 164: 963-5.

NAKAZAWA, S. 1957. Centrifugation de esporas de Equisetum. Anal. Inst. Biol. Univ. Mexico 28: 11-15.

NAKAZAWA, S. 1961. The polarity theory of morphogenetic fields. Sci. Rep. Tohoku Univ. 4th Ser. 27: 57-92.

NAKAZAWA, S. 1963. Role of the protoplasmic connections in the morphogenesis of the fern gametophytes. Sci. Rep. Tohoku Univ. 4th Ser. 29: 247-255.

NAKAZAWA, S. 1975. Regeneration and polarity in Bryopsis. Bull. Jap. Soc. Phycol. 23: 139-143.

NAKAZAWA, S., and OOTAKI, T. 1961. Polarity reversal in Dryopteris protonema. Naturwissen-schaften 48: 557-8.

NAKAZAWA, S., and OOTAKI, T. 1962. A prepattern to the papilla differentiation in Dryopteris gametophyte. Phyton. (Buenos Aires) 18: 113-20.

NEU, H.C., and HEPPEL, L.A. 1965. The release of enzymes from Escherichia coli by osmotic shock during the formation of spheroplasts. J. Biol. Chem. 240: 3685-3692.

NEWCOMB, E.H., and BONNETT, H.T. 1965. Cytoplasmic microtubules and wall microfibril orientation in root hairs of radish. J. Cell Biol. 27: 575-89.

NICOLIS, G. 1975. Dissipative instabilities, structure, and evolution. Adv. Chem. Physics 29: 29-47.

NICOLIS, G., and PRIGOGINE, I. 1977. Self-organisation in non-equilibrium systems. From dissipative structures to order through fluctuation. John Wiley and Sons, New York.

NICHOLS, H.W., and BOLD, H.C. 1965. Trichosarcina polymorpha gen.et.sp.nov. J. Phycol. 1: 34-8.

NICOLSON, G.L. 1976 . Transmembrane control of the receptors on normal and tumor cells, I. Cytoplasmic influence over cell surface components. Biochem. Biophys. Acta 457. 57-108.

NICOLSON, G.L., POSTE, G., and JI, T.H. 1977. The dynamics of cell membrane organization. From: Poste, G., and Nicolson, G.L. (Eds.) Dynamic Aspects of Cell surface Organization p. 1-73. Elsevier/North-Holland Biomedical Press.

NIEUWKOOP, P.D. 1967. The organisation centre: true field phenomena, their origin and significance.
Acta Biotheoretica 17: 151-177.

NIEUWKOOP, P.D. 1977. Origin and establishment of embryonic polar axes in amphibian development.
Curr. Top. Dev. Biol. 11: 115-32.

*
NUCCITELLI, R., and JAFFE, L.F. 1976. Current pulses involving chloride and potassium efflux relieve excess pressure in Pelvetia embryos.
Planta 131: 315-20.

O'BRIEN, T.P., and THIMANN, K.V. 1967. Observations of the fine structure of the oat coleoptile. II. The parenchyma cells of the apex.
Protoplasma 63: 417-42.

OOTAKI, T. 1963. Modification of the developmental axis by centrifugation in Pteris vittata.
Cytologia 28: 21-29.

OXENDER, D.L. 1972. Membrane transport.
Ann. Rev. Biochem. 41: 777-814.

PAUL, D.C., and GOFF, C.W. 1973. Comparative effects of caffeine, its analogue and calcium deficiency on cytokinesis.
Exp. Cell Res. 78: 399-413.

PEASE, D.C. 1939. An analysis of the factors of bilateral determination in centrifuged echinoderm embryos.
J. Exp. Zool. 80: 225-248.

PICKETT-HEAPS, J.D. 1969. Preprophase microtubule bands in some abnormal mitotic cells of wheat.
J. Cell Sci. 4: 397-420.

PICKETT-HEAPS, J.D. 1971. Reproduction by zoospores in Oedogonium. I. Zoosporogenesis.
Protoplasma 72: 275-314.

PICKETT-HEAPS, J.D. 1972. Variation in mitosis and cytokinesis in plant cells : its significance in the phylogeny and evolution of ultrastructural systems.
Cytobios 5: 59-77.

PICKETT-HEAPS, J.D. 1973. Cell division and wall structure in Microspora.
New Phytol. 72: 342-55.

PICKETT-HEAPS, J.D., and FOWKE, L.C. 1969. Cell division in Oedogonium. I. Mitosis, cytokinesis and cell elongation.
Aust. J. Biol. Sci. 22: 857-94.

* NORTHCOTE, D.H. 1971. The golgi apparatus.
Endeavour 109: 26-33.

PICKETT-HEAPS, J.D., and MARCHANT, H.J. 1972. A phylogeny of the green algae: a new proposal.
Cytobios 6: 255-64.

PLANTEFOL, L. 1946. Fondements d'une theorie phyllotaxique nouvelle.
Ann.Sc.Nat.Bot.Serie 11 t7: 153.

PLATT-ALOIA, K.A., and THOMSON, W.W. 1976. An ultrastructural study of two forms of chilling-induced injury to the rind of grapefruit (Citrus paradisis, Mad. Fed.).
Cryobiol 13: 95-106.

POCOCK, M.A. 1960. Hydrodictyon: A comparative biological study.
Jl. S. Afr. Bot. 26: 167-319.

POLLARD, T., and WEIHING, R. 1974. Actin and myosin and cell movement.
CRC Crit. Rev. Biochem. 2: 1-65.

POSTE, G., and ALLISON, A.C. 1973. Membrane fusion.
Biochem. Biophys. Acta 300: 421-65.

POSTE, G., PAPAHADJOPOULOS, D., JACOBSON, K., and VAIL, W.J. 1975a. Effects of local anesthetics on membrane properties. 11. Enhancement of the susceptibility of mammalian cells to agglutination by plant lectins.
Biochem. Biophys. Acta 394: 520-39.

POSTE, G., PAPAHADJOPOULOS, D., and NICOLSON, G.I. 1975b. The effect of local anesthetics on transmembrane cytoskeletal control of cell surface mobility and distribution.
Proc. Natl. Acad. Sci. U.S.A. 72: 4430-4434.

PRESTON, R.D. 1974. The physical biology of plant cell walls.
London: Chapman and Hall.

PRIGOGINE, I. 1947. Étude thermodynamique des Phenomenes Irreverisebles.
Dunod et Liege. Paris.

PRIGOGINE, I., and LEFEVER, R., 1975. Stability and thermodynamic properties of dissipative structures in biological systems. From: Miller, I.R. (Ed.) 1975,
Stability and origin of biological information p. 26-57.
John Wiley and Sons, New York.

QUATRANO, R.S. 1978. Development of cell polarity.
Ann. Rev. Plant Physiol. 29: 487-510.

RACUSEN, R.H., KINNERSLEY, A.M., and GALSTON, A.W. 1977. Osmotically induced changes in electrical properties of plant protoplast membranes.
Science 198: 405-7.

RASMUSSEN, R. 1970. Cell communication, calcium ions, and cyclic adenosine monophosphate.
Science 170: 404-12.

RAY, P.M. 1961. Hormonal regulation of plant cell growth. In D.M.Bonner(Ed.). Control mechanisms in cellular processes pp 155-212 Ronald.N.Y.

READ, H. 1949. The meaning of art.
Penguin Books. England. 197 pp.

REED, P.W., and LARDY, H.A. 1972. A 23187: a divalent cation ionophore.
J. Biol. Chem. 247: 6970-7.

REISS, H.D., and HERTH, W. 1979a. Calcium ionophore A 23187 affects localized wall secretion in the tip region of pollen tubes of Lilium longiflorum.
Planta 145: 225-32.

REISS, H.D., and HERTH, W. 1979b. Calcium gradients in tip growing plant cells visualized by Chlorotetracyline Fluorescence.
Planta 146: 615-21.

RETALLACK, B., and BUTLER, R.D. 1970. The development and structure of the zoospore vesicle in Bulbochaete hiloensis.
Arch. Mikrobiol. 72: 223-37.

REUTER, L. 1953. A contribution to the cell-physiologic analysis of growth and morphogenesis in fern prothallia.
Protoplasma 42: 1-29.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy.
J. Cell Biol. 17: 208-12.

RICHARDS, F.J., and SCHWABE, W.W. 1969. Phyllotaxis: a problem of growth and form. In: Steward, F.C. (Ed.).
Plant Physiology, A Treatise Vol.A. p.79-116,
Academic Press, N.Y.

RITTER, G. 1911. Über Traumatotaxis und Chemotaxis des Zellkernes.
Z. Bot. 3: 1-42.

ROBARDS, A.W. 1976. Plasmodesmata in higher plants. In: Gunning, B.E.S. and Robards, A.W. (Eds.).
Intercellular Communication in Plants: Studies on Plasmodesmata.
p. 15-57., Springer-Verlag, Berlin.

ROBERTS, K. 1974. The structural development of plant cells. In: Bull, A.T. Lagnado, J.R., Thomas, J.O., and Tipton, K.F. (Eds.).
Companion to Biochemistry p. 367-97.
Longman, London.

ROBERTSON, A., and COHEN, M.H. 1972. Control of developing fields. Ann. Rev. Biophys. Bioeng. 2: 409-464.

ROBINSON, D.G., and CUMMINS, W.R. 1976. Golgi Apparatus Secretion in Plasmolyzed Pisum sativum L. Protoplasma 90: 369-79.

ROBINSON, D.G. , and RAY, P.M. 1973. Separation of the synthesis of cellulose and non-cellulosic wall polysaccharides. Plant Physiol. Abstracts p.59.

ROBINSON, D.G., WHITE, R.K., and PRESTON, R.D. 1972. Fine structure of swarmers of Cladophora and Chaetomorpha. III, Wall synthesis and development. Planta. 107: 131-44.

ROSE, B., and LOEWENSTEIN, W.R. 1971. Junctional membrane permeability. Depression by substitution of Li for extracellular Na, and by long-term lack of Ca and Mg; restoration by cell repolarization. J. Memb. Biol. 5: 20-50.

ROSE, B., and LOEWENSTEIN, W.R. 1975a. Permeability of cell junction depends on local cytoplasmic calcium activity. Nature 254: 250-2.

ROSE, B., and LOEWENSTEIN, W.R. 1975b. Calcium ion distribution in cytoplasm visualized by aequorin: Diffusion in cytosol restricted by energized sequestering. Science 190: 1204-6.

ROSE, B., SIMPSON, I., and LOEWENSTEIN, W.R. 1977. Calcium ion produces graded changes in permeability of membrane channels in cell junction. Nature 267: 625-7.

ROSEN, W.G. 1968. Ultrastructure and physiology of pollen. Ann. Rev. Plant Physiol. 19: 435-462.

*RUBIN, R.P. 1974. Calcium and the Secretory Process. Plenum Press, N.Y.

RUBINSTEIN, B. 1977. Osmotic shock inhibits auxin-stimulated modification and growth. Plant Physiol. 59: 369-371.

RUBINSTEIN, B., MAHAR, P., and TATTAR, T.A. 1977. Effects of osmotic shock on some membrane-regulated events of oat coleoptile cells. Plant Physiol. 59: 365-368.

SACHS, T. 1974. The developmental origin of stomata pattern in Crinum. Bot. Gaz. 135 : 314-8.

* Ross, D. J. S. 1980 MLP user guide. Rothamsted Experimental Station, England.

SACHS, T. 1978 . The development of spacing patterns in the leaf epidermis. Symp. Soc. Dev. Biol. 36: 161-183.

SAUERHEBER, R.D., and GORDON, L.M. 1975. Spin label studies on rat liver plasma membrane: Calcium effects on membrane fluidity. Proc. Soc. Exp. Biol. Med. 150: 28-31.

SAWHNEY, V.K., and SRIVASTAVA, L.M. 1977. Comparative effects of cytochalasin B and colchicine on lettuce seedlings. Ann. Bot. 41: 271-4.

SCHALLER, C.H. 1973. Isolation and characterisation of low molecular weight substances activating head and bud formation in Hydra. J. Embryol. Exp. Morphol. 29: 27-38.

SCHMIEDEL, G., and SCHNEPF, E. 1979a. Side branch formation and orientation in the caulonema of the moss, Funaria hygrometrica : Normal development and fine structure. Protoplasma 100: 367-83.

SCHMIEDEL, G., and SCHNEPF, E. 1979b. Side branch formation and orientation in the caulonema of the moss, Funaria hygrometrica: experiments with inhibitors and with centrifugation. Protoplasma 101: 47-59.

SCHMIEDEL, G., and SCHNEPF, E. 1980. Polarity and growth of caulonema tip cells of the moss Funaria hygrometrica. Planta 147: 405-13.

SCHNEPEL, G.H., HEGNER, D., and SCHUMMER, U. 1974. The influence of calcium on the molecular mobility of fatty acid spin labels in phosphatidylserine and phosphatidylinositol structures. Biochim. Biophys. Acta 367: 67-74.

SCHNEPF, E. & KLUMP, J. 1975. Über die traumatotaktische Bewegung der Zellkerne in Tradescantia - Blättern. III. Wirkung von Hemmstoffen der Proteinsynthese. Biochem. Physiol. Pflanzen 168: 123-9.

SCHNEPF, E., and VOLKMANN, K. 1974. Inhibition of traumatotactic movement of nuclei in Tradescantia leaf epidermis. II. Effects of heavy water, ethionine, $Ca^{++}$ and cyclic AMP. Protoplasma 81: 313-21.

SCHNEPF, E., und VON TRAITTEUR, R. 1973. Über die traumatotaktische Bewegung der Zellkerne in Tradescantia-Blättern. Z. Pflanzenphysiol. 69: 181-4.

SCHOUTE, T.C. 1913. Beitrage zur Blattestellungslehre. Rec. Trav. Bot. Néerl 10: 153-324.

SCHWENDENER, S. 1878. Mechanische Theorie der Blattstellungen. Wilhelm Engelman, Leipzig.

SHIMOMURA, T., and DIJKSTRA, J. 1975. The occurrence of callose during the process of local lesion formation.
Neth. J. Plant Path. 81: 107-21.

SIEVERS, A. 1967. Elektronenmikroskopische Untersuchungen zur geotropischen Reaktion. II. Die polare organisation des normal wachsenden Rhizoids von Chara foetida.
Protoplasma 64: 225-53.

SINGER, S.J., and NICOLSON, G.L. 1972. The fluid mosaic model of the structure of cell membranes.
Science 175: 720-31.

SINNOT, E.W. 1960. Plant Morphogenesis.
McGraw Hill, N.Y.

SINNOT, E.W., and BLOCH, R. 1939. Cell polarity and differentiation in root hairs.
Proc. Natl.Acad. Sci. U.S. 26: 223-7.

SMIDSRØD, O., and HAUG, A. 1972. Dependence upon the gel-sol state of the ion-exchange properties of alginates.
Acta Chemica Scandinavica 26: 2063-74.

SMITH, G.M. 1950. Fresh-water algae of the United States. 2nd Ed.
McGraw-Hill, N.Y.

SMITH, M.M., and McCULLY, M.E. 1977. Mild temperature "stress" and callose synthesis.
Planta 136: 65-70.

SMITH, M.M., and McCULLY, M.E. 1978. A critical evaluation of the specificity of aniline blue induced fluorescence.
Protoplasma 95: 229-54.

SNOW, M., and SNOW, R. 1962. A theory of the regulation of phyllotaxis based on Lupinus albus.
Philos. Trans. R. Soc. Lond. Ser B. 244: 483-513.

SONTAG, W. 1977. A comparative kinetic study on the conversion of fluoresceindiacetate to fluorescein in living cells and in vitro.
Rad. and Environm. Biophys. 14: 1-12.

SPURR, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy.
J. Ultrastruct. Res. 26: 31-49.

STANGE, L. 1957. Untersuchungen über die Umstimmungs - und Differenzierungsvorgänge in regenerierenden Zellen des Lebermooses Riella.
Z. Bot. 45: 197-244.

STEINHARDT, R.A., and EPEL, C.D. 1974. Activation of sea urchin eggs by a calcium ionophore.
Proc. Natl.Acad. Sci. U.S.A. 71: 1915-9.

STETLER, D.A., and DE MAGGIO, A.E. 1972. An ultrastructural study of fern gametophytes during one- and two-dimensional development.
Am. J. Bot. 59: 1011-7.

STEVENS, P.S. 1976. Patterns in Nature.
Penguin Books, England.

STEWART, W.D.P. 1973. Nitrogen fixation. In: Carr, N.G., and Whitton, B.A. (Eds.).
The Biology of blue-green algae. p. 260-78.
Blackwell Scientific Publications, Oxford.

STEWART, K.D., and MATTOX, K.R. 1975. Comparative cytology, evolution and classification of the green algae with some consideration of the origin of other organisms with chlorophylls A and B.
Bot. Rev. 41: 104-35.

STEWART, K.D., MATTOX, K.R., and FLOYD, G.L. 1973. Mitosis, cytokinesis and distribution of plasmodesmata and other cytological characteristics in the Ulotrichales, Ulvales and Chaetophorales-phylogenetic and taxonomic considerations.
J. Phycol. 9: 128-41.

SUSSEX, I.M. 1952. Regeneration of the potato shoot apex.
Nature 170: 755-7.

SUSSEX, I.M. 1972. Do concepts of animal development apply to plant systems?
Brookhaven Symp. Biol. 25: 145-51.

TANNENBAUM, J., TANENBAUM, S.W., and GODMAN, G.C. 1977a. The binding sites of cytochalasin D. I. Evidence that they may be peripheral membrane proteins.
J. Cell Physiol. 91: 225-38.

TANNENBAUM, J. TANENBAUM, S.W., and GODMAN, G.C. 1977b. The binding sites of cytochalasin D.II. Their relationship to hexose transport and to cytochalasin B.
J. Cell Physiol. 91: 239-48.

TANNENBAUM, J., TANENBAUM, S.W., LO, L.W., GODMAN, G.C., and MIRANDA, A.F. 1975. Binding and subcellular localization of tritiated cytochalasin D.
Exp. Cell Res. 91: 47-56.

TILNEY, L.G., and DETMERS, P. 1975. Actin in erythrocyte ghosts and its association with spectrin. Evidence for a nonfilamentous form of these two molecules in situ.
J.Cell Biol. 66: 508-20.

TIPPIT, D.H., and PICKETT-HEAPS, J.D. 1974. Experimental investigations into morphogenesis in Micrasterias.
Protoplasma 81: 271-96.

TORREY, J.G., and GALUN, E. 1970. Apolar embryos of Fucus resulting from osmotic and chemical treatment.
Am. J. Bot. 57: 111-9.

TRIGGLE, D.J. 1972. Effects of calcium on excitable membranes and neurotransmitter action, In: Danielli, J.F., Rosenberg, M.D., and Cadenhead, D.A. (Eds.).
Progress in Surface and Membrane Science, Vol.5. p. 267-331.
Academic Press, N.Y.

TRUESDELL, L.C., and CANTINO, E.C. 1971. The induction and early events of germination in the zoospore of Blastocladiella emersonii.
Curr. Top. Dev. Biol. 6: 1-44.

TURING, A.M. 1952. The chemical basis of morphogenesis.
Phil. Trans. Roy. Soc. B. 237: 37-72.

TYREE, M.T. 1970. The symplast concept. A general theory of symplastic transport according to the thermodynamics of irreversible processes.
J. Theor. Biol. 26: 181-214.

UNANUE, E.R., KARNOVSKY, M.J., and ENGERS, H.D. 1973. Ligand-induced movement of lymphocyte membrane macromolecules, III. Relationship between the formation and fate of anti-Ig surface Ig complexes and cell metabolism.
J. Exp. Med. 137: 675-89.

USPENSKAIA, V.I. 1936. The influence of lighting intensity and concentration of nitrates on the development of the thallome and the formation of zoospores and gametes in Stigeoclonium tenue.
Klebsi Microbiol. 5: 307-321.

VAN ITERSON, G. 1907. Mathematische and mikroskopisch-anatomische Studien uber Blättstellungen.
Gustav Fischer, Jena.

VEEN, A.H., and LINDENMAYER, A. 1977. Diffusion mechanism for phyllotaxis.
Plant Physiol. 60: 127-39.

VISCHER, W. 1933. Über einige Kritische Gattungen und die Systematik der Chaetophorales.
Bot. Centralbl. 51: 1-100.

VON BERTALANFFY, L. 1952. Problems of Life:An Evaluation of Modern Biological Thought.
Wiley, New York.

WADDINGTON, C.H. 1966. Fields and gradients.
Symp. Soc. Dev. Biol. 25: 105-24.

WAGNER, G., and BELLINI, E. 1976. Light-dependent fluxes and compartmentation of calcium in the green alga Mougeotia.
Z. Pflanzenphysiol. 79: 283-91.

WALKER, N.A. 1976. Transport of solutes through the plasmodesmata of Chara nodes. In: Gunning, B.E.S., and Robards, A.W. (Eds.) Intercellular Communication in Plants: Studies on Plasmodesmata. p. 165-79. Springer-Verlag, Berlin.

WARDLAW, C.W. 1949. Experiments on organogenesis in ferns.
Growth (Suppl.) 13: 93-131.

WARREN WILSON, J. 1978. The position of regenerating cambia ; auxin/surcrose ratio and the gradient induction hypothesis.
Proc.Roy.Soc.Lond.Ser.B. 203: 153-76.

WARREN WILSON, J.M. and WARREN WILSON, P.M. 1961. The position of regenerating cambia - a new hypothesis.
New Phytol. 60: 63-73.

WATERKEYN, L. 1967. Sur l'existence d'un "stade callosique" présenté par la paroi cellulaire, au cours de la cytocinèse.
C.R.Acad.Sci.(Paris) 265: 1792-4.

WEBER, W. 1958. Zur Polarität von Vaucheria.
Z.Bot. 46: 161-198.

WEBER, A. 1968. The mechanism of the action of caffeine on sarcoplasmic reticulum.
J. Gen. Physiol. 52: 760-2.

WESSELS, N., SPOONER, B., ASH, J., BRADLEY, M., LUDUENA, M., TAYLOR, E., WRENN, J., and YAMADA, K. 1971.
Microfilaments in cellular and developmental processes.
Science 171: 135-43.

WETMORE, R.H., and RIER, J.P. 1963. Experimental induction of vascular tissues in callus of angiosperms.
Am. J. Bot. 50: 418-30.

WHITAKER, D.M. 1937. Determination of polarity by centrifuging eggs of Fucus furcatus.
Biol. Bull. 73: 249-260.

WIGGLESWORTH, V.B. 1940. Local and general factors in the development of "pattern" in Rhodnius prolixus (Hemiptera).
J. Exp. Biol. 17: 180-200.

WILCOX, M., MITCHISON, G.J., and SMITH, R.J. 1973a. Pattern formation in the blue-green alga Anabaena. I. Basic mechanisms. J. Cell Sci. 12: 707-23.

WILCOX, M., MITCHISON, G.J., and SMITH, R.J. 1973b. Pattern formation in the blue-green alga Anabaena. II. Controlled proheterocyst regression. J. Cell Sci. 13: 637-49.

WILCOX, M., MITCHISON, G.J., and SMITH, R.J. 1975. Spatial control of differentiation in the blue-green alga Anabaena. Microbiology 1975:453-63.

WILEY, W.R. 1970. Trytophan transport in Neurospora crassa: a tryptophan-binding protein released by cold osmotic shock. J. Bacteriol. 103: 656-62.

WILLIS, R.C., and FURLONG, C.E. 1974. Purification and properties of a ribose-binding protein from Escherichia coli. J. Biol. Chem. 249: 6926-9.

WILSON, L., and FRIEDKIN, M. 1967. The biochemical events of mitosis. The in vivo and in vitro binding of colchicine in grasshopper embryos, and its possible relation to inhibition of mitosis. Biochemistry 6: 3126-3135.

WILSON, O.H., and HOLDEN, J.T. 1969. Arginine transport and metabolism in osmotically shocked and unshocked cells of Escherichia coli W. J. Biol. Chem. 244: 2737-42.

WOLK, C.P. 1967. Physiological basis of the pattern of vegetative growth of a blue-green alga. Proc. Natl. Acad. Sci. U.S.A. 57: 1246-51.

WOLK, C.P. 1975. Differentiation and pattern formation in filamentous blue-green algae. In: Gerhardt, P., Sadoff, H., and Costilow, R. (Eds.) Spores VI. p. 85-96. American Society for Microbiology, Washington, D.C.

WOLPERT, L. 1969. Positional information and the spatial pattern of cellular differentiation. J. Theor. Biol. 25: 1-47.

WOLPERT, L. 1971. Positional information and pattern formation. Curr. Top. Dev. Biol. 6: 183-224.

WORLEY, J.F. 1968. Rotational streaming in fibre cells and its role in translocation. Pl. Physiol. 43 : 1648-1655.

WU, J.H., and DIMITMAN, J.E. 1970. Leaf structure and callose formation as determinants of TMV movement in bean leaves as revealed by UV irradiation studies.
Virology 40: 820-7.

YAHARA, I., and EDELMAN, G.M. 1972. Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A.
Proc. Natl. Acad.Sci. U.S.A. 69: 608-12.