MICROTUBULES AND MORPHOGENESIS IN

AZOLLA PINNATA ROOTS

BY

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FIG. 1.1  *Azolla pinnata*, Bega, N.S.W.
STATEMENT

All work reported in this thesis is original and my own.

Albertine Harehahn
Anyone who undertakes the task of cutting thousands of microtubules into little pieces and then putting them back together again is likely to run into problems. However, with the help of many people within R.S.B.S. and others in the C.S.I.R.O. not only were these problems resolved, but also the three years of my PhD were made very enjoyable.

Firstly, I must express gratitude to Professor D.J. Carr for allowing me the opportunity to carry out this work in his laboratories in the Department of Developmental Biology, R.S.B.S. I thank Dr. D.J. Goodchild and Mr. S. Craig of the C.S.I.R.O. for their continued interest, help and encouragement. It was only through the aid of the staff of the EM Unit, R.S.B.S., that I was able to obtain the thousands of micrographs required for the task, and then through the patience and friendship of those in the Department of Developmental Biology was I able to maintain my sanity while "putting the pieces back together"!

My handwriting has been deciphered and beautifully typed by Mrs. S. Cupit, to whom I give many thanks for her keen interest in the presentation of this thesis. To Dr. I.G. Manning, who offered to check that I had spelt "microtubule" correctly 2,911 times, I am very much indebted.

Finally, it is with great sincerity that I thank Professor B.E.S. Gunning for his excellent supervision throughout my PhD. I acknowledge the receipt of a Commonwealth Postgraduate Research Award.
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ABSTRACT

The root of the water fern, _Azolla pinnata_ was shown to be very advantageous for studies of morphogenesis using ultrastructural approaches. After characterising the system a detailed examination was made of the microtubule arrays which occur in the cortex of plant cells.

The root has all of the cellular complexities of any vascular axis but it has been possible to analyse the development of the entire root structure in terms of the lineages that generate each cell type in the zone of formative divisions and the subsequent transverse proliferative divisions along the cell files. The evidence indicates that the sites of proliferative divisions are related to the cell dimensions, but that cell cycle durations and the positions at which cell differentiation events occur vary, being dependent on root age.

In _Azolla_, as in other roots, transversely aligned, cortical microtubules lie adjacent to the longitudinal cell walls. Sequences of serial sections showing transverse views of microtubules were used to track individual tubules and revealed that cortical microtubules are not continuous hoops but are short relative to the cell circumference. The cortical arrays at interphase, prophase and those overlying developing xylem thickenings consist of overlapping component microtubules which are cross-bridged to the plasma membrane and to adjacent
microtubules. This analysis provides an explanation for the disparity in numbers of microtubules that have been observed on opposite sides of cells. The concept that emerges is one of a dynamic array, the form of which might be controlled by additions, deletions, or changes in length of the constituent microtubules. Treatments which can cause microtubule depolymerisation or enhance polymerisation served to emphasise the dynamic property of the arrays and the effects of such treatments were documented.

The detailed knowledge of the construction of the root enables the sites and planes of cell division to be predicted and pre-prophase bands of microtubules were found to occur at the predicted sites prior to every category of division in the root, accurately anticipating the site of fusion of the future cell plate with the parental walls. The number of microtubules in the band varies, and may be a function of the stage of development of the band and the size of the cell.

Intercalation of microtubules throughout interphase arrays maintains the density (number per unit length of wall) of microtubules during cell elongation. The density can be characteristic not only of the cell type but also of individual walls in a given cell type. Increased wall deposition during differentiation of sieve and xylem elements was found to be preceded by an increase in the numbers of cortical microtubules in the cells. The distribution of microtubules implies a capacity to initiate
microtubule assembly along the length of the cell. No strong evidence of microtubule organising regions along the faces of cells was observed in the sequences of serial sections, but supportive evidence for an hypothesis stating that microtubules are initiated along cell edges was obtained in roots which were re-establishing their cortical arrays following colchicine treatment.
CHAPTER 1
CORTICAL MICROTUBULES IN PLANT CELLS

1.1 INTRODUCTION

One of the many components of cells that were revealed by the high resolving power of the electron microscope was a system of hollow cylinders, of undefined length, of outer diameter 18-27nm and wall thickness 5-7nm. In reviewing the numerous and diverse observations that had accrued by 1963, Slautterback commented that one of the most striking features of these "microtubules", the descriptive term he adopted, was their ubiquity. They had by then been seen in a wide variety of animal cells and similar structures had been described in the spermatozoids of a moss (Manton, 1957) and a fern (Manton, 1959). However, the full extent of their distribution was not realized until the observation of similar microtubules lying in the cortical cytoplasm and mitotic spindle of plant root tip cells, by Ledbetter and Porter (1963).

Since 1963, much has been learned about these cell components which have been found to occur in virtually all eukaryotic cells, both plant and animal, at some stage of their growth and development. They constitute the structural elements in cilia and flagella, in the axopodia and oral feeding apparatus of several protozoans, in centrioles and basal bodies and in both meiotic and mitotic spindles. They are involved in the formation and
maintenance of specific cell shapes in animal cells and in those plant cells which lack a rigid, external cell wall. In plant cells, microtubules comprise the phragmoplast fibres during cell plate formation and occur in the cell cortex during primary and secondary wall formation and in a transitory, cortical band before prophase. In some of these situations the microtubules may play a purely structural role but in others there is evidence that they are directly involved with motility of, or within, the cell either by sliding relative to one another or by their interaction with other cellular components.

The organisation and function of microtubules in the cortex of higher plant cells and their role in plant cell morphogenesis, form the primary concern of this thesis. The spatial and temporal distribution of cortical microtubules suggests an involvement in each of the three important aspects of plant cell morphogenesis, namely: cell division, cell expansion, and cell differentiation. The transitory band of microtubules which forms before prophase precisely predicts the site of fusion of the new cell plate with the parental walls following cell division. Through their influence on the orientation of the cellulose microfibrils in the cell wall, microtubules are thought to regulate the direction of cell expansion and the shapes of the cells. Cell differentiation, in many cases, incorporates the development of specific wall patterns and properties and again, cortical microtubules are considered to be involved with the ordered deposition of the cellulosic wall material.

The aim of this introductory chapter is to provide
a background relevant to the studies of cortical microtubules described in this thesis. A number of recent reviews cover more general aspects of microtubules including: microtubule structure and biochemistry (Olmsted and Borisy, 1973; Wilson and Bryan, 1974; Snyder and McIntosh, 1976), microtubules in animal cells (Roberts, 1974) and plant microtubules (Newcomb, 1969; Hepler and Palevitz, 1974; Pickett-Heaps, 1974; Hepler, 1976). A brief description of microtubule substructure and assembly and the ability of microtubules to serve a cytoskeletal function in naked plant cells will be given before discussing the literature relating to cortical microtubules in higher plant cells, i.e. the microtubules lying in the cell cortex adjacent to the cell wall.

1.2 Microtubule Structure and Assembly

The wall of the microtubule has an outer diameter of approximately 24nm and is composed of a number of protofilaments, usually 13, which are made up of rows of approximately globular subunits 4-5nm in diameter. Tubulin, the protein subunit of microtubules, has been isolated from a wide variety of sources and found to consist of a dimer which yields two monomers of approximately 55,000 M.W. The monomers are considered to be equivalent to the globular subunits comprising the protofilaments of the microtubule wall. Plant microtubules are thought to be similar to those of animal origin, although little is known about plant tubulin (Hart and Sabnis, 1976).

A dynamic equilibrium exists between free tubulin
subunits and those incorporated into intact microtubules. Low temperatures and high pressures cause a shift in the equilibrium towards the disassembled state while warmer temperatures or deuterium oxide promote microtubule assembly. The antimitotic drug, colchicine, binds to the tubulin dimers and prevents their polymerisation, resulting in the disassembly of the microtubules. Arms or cross-bridges 2-5 nm in width and 5-40 nm in length are often seen in association with the outer surface of the microtubules. Functions in motility, in determining precise arrangement of microtubules, or as stabilising elements, have been proposed for the cross-bridges. Microtubule assembly appears to occur at initiating sites or "microtubule-organising centres" (MTOCs), which are characterised by an amorphous electron-dense material. In plant cells such regions have been identified at kinetochores, in the midzone of the phragmoplast, and in the cortex of some algal cells which lack a cell wall. The microtubule arrays arising from the latter MTOCs apparently serve a function that differs from that of the cortical microtubule arrays in higher plant cells, and are described briefly below.

1.3 Cytoskeletal Microtubules

The initial observations of Ledbetter and Porter (1963) have been followed by many attempts to elucidate the organisation and function of cortical microtubule arrays in plant cells. Porter (1966) suggested that the microtubules might form a cytoskeleton to shape the cell protoplast and to keep selected zones of the cell surface in
intimate contact with the external wall. However, a direct cytoskeletal function such as this is not generally envisaged for microtubule arrays in plant cells which are encased in a relatively rigid cell wall, but does appear to be likely in many plant cells which lack such a wall. For instance, the unicellular, biflagellate alga *Ochromonas*, is maintained in a tear drop shape by two sets of microtubules. One set, the kineto-beak microtubules, forms a platform at the anterior end of the cell, and a second set, the rhizoplast microtubules, extends into, and shapes the tail (Bouck and Brown, 1973). Treatment of the cells with colchicine solutions or hydrostatic pressure leads to the disappearance of the microtubules and loss of cell shape (Brown and Bouck, 1973). The two sets of microtubules show different sensitivities to these treatments, the rhizoplast microtubules being the first to depolymerize during treatment and the last to reappear on recovery.

A cytoskeletal role has also been proposed for bands of microtubules underlying the plasmalemma of a number of net-forming algal zoospores (Marchant, 1974a, 1974b; Marchant and Pickett-Heaps, 1974; Millington and Gawlik, 1975). The microtubules are involved in changing the shape of the zoospores before and during aggregation to form a net-like colony, and, in *Pediastrum*, in the formation of horns on peripheral cells. Treatment with colchicine leads to loss of the microtubules and the failure of the zoospores to aggregate in an ordered array, or to form horns (Marchant and Pickett-Heaps, 1974). The gametes and zoospores of a number of green algae have been grouped into two categories
of cell shape, based in part on different dispositions of cytoskeletal microtubules (Pickett-Heaps, 1975). Bundles of cytoplasmic microtubules performing a cytoskeletal function have also been described during spermiogenesis in higher plants (Cass and Karas, 1975; Myles and Hepler, 1977) and in the elongated generative cell of *Endymion non-scripta* (Burgess, 1970a,c).

A cytoskeletal function for cortical microtubules in plant cells which do possess a cell wall has been recently suggested by Schnepf (1973, 1974). The mechanism envisaged, however, is the reverse of that initially suggested by Porter (1966). The microtubules are thought to allow localised wall thickening by lifting the plasma membrane off the wall, against the turgor pressure, forming an "extracytoplasmic space" in which wall material can be accumulated. Before discussing this proposal further, the occurrence and disposition of cortical microtubules in plant cells which possess an external cell wall will be examined in detail.

1.4 CORTICAL MICROTUBULES AND WALL FORMATION

1.4.1 Introduction

The diversity of cell sizes and shapes found in multicellular plant tissues is well illustrated in either longitudinal or transverse sections of *Azolla pinnata* roots (e.g. Figs. 2.9-2.18). The dimensions of every cell in the root are defined by the cell wall which surrounds the protoplast, lying external to the plasma membrane.
Protoplasts of *Azolla* have not been made, but in other tissues where the cell walls have been removed by enzymatic digestion, it is clearly demonstrated that the diversity of cell shape is thereby lost, all cells reverting to a spherical shape through the no longer restricted action of surface tension forces. The cell wall is a mould, fully occupied in turgid conditions by the protoplast. Unlike most moulds, however, the contents are responsible for its fabrication so that, when stressed by turgor forces, it will assume a particular shape.

Different cell shapes are a reflection of distinctive wall properties. Although the great majority of walls consist of an amorphous matrix of carbohydrates reinforced by microfibrils of cellulose or chitin, there are widely different textures and chemical compositions. In an actively growing root, for example, the majority of walls near the root tip will be thin, primary walls. Their deposition accompanies rapid cell expansion, the proportion of cellulose being less than that of the other wall carbohydrates (Roelofsen, 1959; Frey-Wyssling and Mühlethaler, 1965). In other cells, particularly those in the more mature part of the root, elongation may have ceased and the cells will be participating in the formation of secondary walls in which cellulose predominates over the other wall constituents. In some cases both primary and
secondary wall formation can occur simultaneously in the same cell, for example in differentiating xylem elements. The overall length of these cells may be increasing, but secondary wall deposition is considered to occur at localised zones of the wall which are not expanding. Another type of wall deposition will be occurring in the root hairs which grow by a mechanism involving the addition of new wall material at the tip of the filament.

Microtubules have been observed in the cortical cytoplasm during all three categories of wall deposition. In their initial description, Ledbetter and Porter (1963) noted that the alignment of the cortical microtubules paralleled that of the microfibrils in the adjacent primary wall and they suggested that the microtubules might influence the orientation of the wall microfibrils. The observations of cortical microtubule and wall microfibril dispositions that have followed, generally support this contention and will be reviewed below.

1.4.2 Tip Growth

Some cells are characterised by extreme elongation and their growth in surface area is largely confined to the tip of the usually filamentous structure. These include root hairs, pollen tubes, fungal hyphae and fern and moss protonemata. Cotton fibres also engage in tip growth but extension growth occurs along the sides as well as the tip and will not be included in this section.
Rapidly growing radish root hairs have two distinct layers of wall microfibrils: an outer layer of randomly arranged microfibrils which arise at the tip and an inner layer of axially aligned microfibrils which are first visible about 25µm from the tip (Newcomb and Bonnett, 1965). The cortical microtubules in the root hairs are also axially aligned, the array extending from the base of the hair to within 2 to 3 µm of the tip. The microtubules thus appear in an organised array about 20µm in advance of the organised wall microfibrils a separation in time equivalent to 12 to 15 minutes.

The presence of microtubules in pollen tubes was reported for the first time in 1972 in *Lilium* and *Clivia* pollen tubes (Franke et al., 1972). Many microtubules underlying the plasma membrane were axially aligned but other cortical microtubules, often in groups of 5 or 6, passed at acute angles to the longitudinal axis of the pollen tube. Microtubules were absent from the extreme apical zone, i.e. approximately 2µm from the tip. Extensive cross-bridging between microtubules and between microtubules and the plasma membrane was observed. Broad strands of microfilaments were seen between the microtubules. Treatments with colchicine which led to the complete disappearance of the microtubules did not affect either the germination of the pollen or cytoplasmic streaming and tip growth of the elongating pollen tubes. Tip growth and cytoplasmic
streaming were both sensitive to treatment with cytochalasin B, suggesting the involvement of the microfilamentous structures in these processes (Franke et al., 1972).

In the pollen tube walls of *Lilium* and *Petunia* the cellulose microfibrils are reported to be oriented in two directions both at angles of approximately 45° to the main axis of the tube (Rosen, 1968). The orientation of the microfibrils over the tip was not ascertained in *Lilium* but in *Petunia* they were randomly arranged.

In vegetative fungal hyphae microtubules appear to be abundant in all species adequately examined (Heath, 1975a). Based on morphological evidence only, the microtubules were suggested to be involved in organelle motility, including a possible track-like function in the movement of vesicles containing wall material (Heath et al., 1971). Axially aligned microtubules are also present in the rhizoids of germinating zoospores of *Blastocladiella* (Barstow and Lovett, 1974).

The spores of most species of ferns require some exposure to light before they will germinate. If subsequently cultured in the dark plants will grow as long, filamentous protonemata with a low rate of cell division. Germination and filamentous growth is promoted by red light, but blue or white light causes lateral expansion of the filament tip, active cell division and the transition to a
two-dimensional prothallus (Miller and Stephani, 1971). If filamentous protonemata are grown in the dark or under red or far red light on media containing colchicine only slight expansion occurs. If irradiated with blue or white light in the presence of colchicine, there is no cell division but extensive lateral expansion of the apical and subapical cells of the filament results in a large, spherical tip (Miller and Stephani, 1971; Stockwell and Miller, 1974).

The filamentous protonemata grown in red light contain two sets of microtubules. One set is aligned parallel to the long axis of the protonema extending to approximately 20µm from the apex (Stetler and DeMaggio, 1972) or almost to the region where tip curvature begins (Wada and O'Brien, 1975). A second, more apical set of microtubules reaching to within approximately 5µm of the tip is reported to consist of randomly oriented microtubules (Stetler and DeMaggio, 1972) or of circumferentially aligned microtubules (Wada and O'Brien, 1975). Following irradiation by white or blue light, the microtubules were reduced in number throughout the apical cell and did not display any preferred orientation (Stetler and DeMaggio, 1972; Wada and O'Brien, 1975). Wall microfibrils seemed to possess the same orientation as the microtubules in plants grown in either red or white light (Stetler and DeMaggio, 1972).
1.4.3 Primary Walls

The importance of the cellulose microfibrils in determining the manner of plant cell enlargement was expounded by Green in 1962. He viewed growth of the plant cell as the yielding of the cell wall to internal turgor pressure. In cylindrical cells growth along the cell axis is the result of anisotropic yielding of the wall due to strong transverse reinforcement by transversely oriented cellulose microfibrils. He proposed that the orientation of these microfibrils was controlled by proteinaceous elements in the cortical cytoplasm, similar to spindle fibres. Ledbetter and Porter's report of cortical microtubules in elongating root tip cells appeared in the following year. In these cells, arrays of "hoop-like", transversely aligned microtubules lay against the longitudinal walls, mirroring the orientation of the cellulose microfibrils in the adjacent wall (Fig. 1.2A).

Both Newcomb (1969) and O'Brien (1972) noted that there were very few reports of attempts to confirm or extend the initial (Ledbetter and Porter, 1963) observations of microtubule dispositions at primary cell walls, indeed, some comments made in passing even appeared to be contradictory (see O'Brien, 1972). In many cases, the microtubule orientation is correlated with the orientation of electron-dense fibrils in the wall which are thought to be cellulosic microfibrils. However, O'Brien
(1972) has drawn attention to the inability of heavy metal stains (uranyl and lead salts) used in staining ultra-thin sections for electron microscopy to react with cellulose (Heyn, 1966; O'Brien, 1970). It has since been found that these stains can be removed by washing and a physical mechanism rather than a chemical reaction may be involved (Cox and Juniper, 1972). Wherever cellulosic microfibril orientation has been determined using polarised light microscopy (e.g. Palevitz and Hepler, 1976) it is the same as that exhibited by the fibrils seen in thin sections with the electron microscope.

Since the reviews by Newcomb (1969) and O'Brien (1972) additional observations of the occurrence of cortical microtubules during primary wall deposition have been made. In some cases, the alignment of the wall microfibrils and that of the cortical microtubules is set, but in others the orientation of these components may change during development. Some studies have also included observations of the effects of treatments which influence microtubule disposition and cell growth.

The Alignment of Wall Microfibrils and Cortical Microtubules:

The description of transversely aligned microtubules along the longitudinal walls in elongating root tip cells of *Phleum*, *Spirodela*
and *Juniperus* seen in thin sections (Ledbetter and Porter, 1963) was extended by the observation of cortical microtubules with the same type of orientation in freeze-etched preparations of *Pisum* root tip cells (Northcote and Lewis, 1968). Transversely oriented microtubules have also been observed in elongating parenchyma cells of *Nymphoides petioles* (Freundlich, 1974).

Microtubules have been observed in both resting and active cambium of *Pinus* (Srivastava and O'Brien, 1966a), *Fraxinus* (Srivastava, 1966) and *Salix* (Robards and Kidwai, 1969). In the fusiform initials of *Pinus* and *Fraxinus* there are two sets of microtubules, one oriented parallel to the long axis of the cell, the other running circumferentially around it. In sections showing the orientation of both the cortical microtubules and recently deposited wall microfibrils, a parallel alignment was revealed. The microtubule orientation was more irregular in the ray initials of *Pinus*. In the resting cambium of *Salix*, the arrays of microtubules were longitudinally oriented, but their alignment in the active cambium was not described.

*Sphagnum* leaflets are only one cell layer in thickness and develop by symmetrical and asymmetrical anticlinal divisions (Schnepf, 1972, 1973, 1974). During interphase, microtubules are found exclusively near the cell walls, often connected by bridges to the plasma membrane and are oriented
anticlinally along the inner walls but are "dispersed" on the outer walls. Cellulose microfibrils are reported to be randomly distributed in all primary walls. Immediately following division an uneven distribution of microtubules is observed, most lying adjacent to the new wall. For this analysis median sections were cut parallel to the surface of the leaflets and it is not clear how the microfibril orientation in the anticlinal walls was determined. Parallel alignment between microtubules and wall microfibrils has been reported in the meristematic gametophyte tissue of the moss, Funaria, and the liverwort, Riella (Schulz and Lehmann, 1970). Transversely oriented wall microfibrils and cortical microtubules have been observed in Chara (Pickett-Heaps, 1967a), Spirogyra (Fowke and Pickett-Heaps, 1969) and Nitella (Green, 1969).

Changing Systems:

In the elongation phase of cotton fibre development, the primary wall is composed of several layers which spiral along the length of the fibre axis in alternating alpha and beta helices (Westafer and Brown, 1976). The cortical microtubules were reported to be sparse and generally oriented parallel to the long axis of the fibre. This distribution may reflect the change of the microtubule arrays accompanying the change in microfibril orientation.
Cortical microtubules have been observed in both proembryonic and embryonic cells of cultured carrot tissues (Wochok, 1973). The transition in developmental pattern from proembryonic to embryonic cells was accompanied by a change in orientation of the microtubules and wall microfibrils from a random distribution, especially in peripheral cells, to one that is ordered.

The cell wall of the alga, Oocystis, contains a high proportion of cellulose and consists of 20-30 lamellae of highly ordered microfibrils. The microfibrils are parallel to one another, their orientation changing by 90° in adjacent lamellae (Robinson et al., 1976; Sachs et al., 1976). The two microfibril directions form helices which close in at the two poles of the ellipsoidal cells, and lie at 60° and 30° to the major axis of the cell. The microfibrils have a diameter of approximately 12nm and are recognizable in electron microscope preparations. Microtubules in the cell cortex always lie parallel to one or other of the microfibril directions. The numerical ratio of microtubules to microfibrils was estimated to be 1:2.5.

The flagellate alga, Poteriochromonas stipitata [syn. Ochromonas malhamensis] (Schnepf, 1974; Schnepf et al., 1975) lacks a cell wall but on attaching to a suitable substrate the cells form a protoplasmic tail, similar to that of Ochromonas danica (Bouck and Brown, 1973), stiffened by a
helical band of microtubules which are cross-bridged to the plasmalemma. A chitinous lorica is then formed, the stalk of which, when completed, consists of about 16 main fibrils running in steep helices (Schnepf et al., 1975; Herth et al., 1977). Smaller secondary microfibrils lie internal to the main fibres. The chitin microfibrils of the lorica are formed at the surface of the plasmalemma, elongate unidirectionally by tip growth, and aggregate into the ribbon-like primary fibrils. These latter fibrils have a width of approximately 20 nm and every primary fibril coincides precisely with a microtubule in the tail.

Experimental Treatments:

The descriptions of the disposition of microtubules during primary wall deposition have, in some cases, been coupled with investigations of the effects of chemical and physical treatments which affect microtubules and cell growth. These experimental studies have greatly contributed to the understanding of the involvement of cortical microtubules in wall formation.

Treatment of the growing cylindrical cells of Nitella (Green, 1969) and Chara (Pickett-Heaps, 1974) with colchicine leads to the loss of the transversely oriented microtubules. Newly deposited microfibrils are randomly oriented and the cells swell laterally. Even when lateral expansion of the
colchicine-treated *Nitella* cells is physically restrained, the orientation of newly deposited microfibrils remains random. On removal of the colchicine, deposition of transversely oriented microfibrils resumes (Green, 1969; Gertel and Green, 1977).

Treatment of *Oocystis* with colchicine results in depolymerisation of the microtubules and the loss of the wall pattern (Robinson *et al*., 1976; Grimm *et al*., 1976). Colchicine does not alter the amount of cellulose deposited in the wall but the wall develops without the alternation in orientation of the adjacent lamellae. The microfibrils are parallel, often arranged in bundles and there are areas of wall where no microfibrils can be seen. On removal of colchicine (if the treatment was not longer than 24 hours) a recovery was obtained, and the change of microfibril orientation in adjacent lamellae resumed.

In the studies of the alga *Poteriochromonas* it was found that treatment with colchicine caused depolymerisation of the microtubules and the failure of the tail to form normally. The lorica was still produced but had a shorter stalk or only consisted of the upper cup. In these circumstances, the wall of the upper cup appeared thicker, presumably through the deposition of material that would have formed the stalk (Schnepf, 1974; Schnepf *et al*., 1975).
Treatment of growing root tips of *Phleum* with colchicine solutions led to the loss of normally transversely oriented microtubules and a change from axial growth to an isodiametric expansion of the root tip cells (Brennan, 1970).

Colchicine treatments have also been used to investigate the role of microtubules in hormone-induced cell elongation in lettuce and bean hypocotyls. The epidermal and some cortical cells in lettuce hypocotyls have thick polylamellate walls in which lamellae with longitudinal orientation of microfibrils alternate with those having transverse orientation of microfibrils. The cortical microtubules generally lie parallel to the inner-most wall microfibrils (Sawhney and Srivastava, 1975). When cell elongation is increased by gibberellic acid treatment, the arrangement of microtubules and inner-most wall microfibrils is predominantly transverse in the cortical cells and longitudinal in the epidermal cells. These results were taken to suggest that the cortical cells were involved in control of the directionality of growth of the hypocotyl.

In the epidermal cells of bean hypocotyls, microtubules are randomly oriented along the tangential walls and transversely arranged along the radial walls (Shibaoka, 1974). Treatment with indolacetic acid (IAA) initially produced cell elongation (without an increase in cell division) and at a later period of incubation, lateral cell
expansion. The orientation of the epidermal cell microtubules was not changed by IAA. IAA-induced cell elongation was not affected by colchicine in bean (Shibaoka, 1974) or lettuce hypocotyls (Sawhney and Srivastava, 1977). However, following gibberellic acid treatment all microtubules in the bean epidermal cells were transversely aligned. Colchicine treatment inhibited gibberellic acid-induced cell elongation in both lettuce (Sawhney and Srivastava, 1974; Srivastava et al., 1977) and bean hypocotyls (Shibaoka, 1972). In lettuce, colchicine was found not to inhibit the kinetics of growth nor to decrease the amount of wall material deposited but its application did lead to a loss of cortical microtubules. The cells became spherical and the precise arrangements of wall microfibrils were also totally lost in the gibberellic acid and colchicine treated material. Even though they showed some degree of order, the inner-most microfibrils did not exhibit any predominant orientation but along the same wall could vary from transverse to oblique or longitudinal (Srivastava et al., 1977).

Treatment of bean hypocotyls with kinetin or benzimidazole (BIA) inhibited cell elongation and increased cell expansion. Both treatments changed the orientation of the epidermal cell microtubules to a predominantly longitudinal arrangement. Colchicine alleviated the kinetin and BIA inhibition of cell elongation (Shibaoka, 1974).
Treatment of pea internodes with the auxin analogue 2,3,5-triiodobenzoic acid (TIBA) resulted in proliferation of the plasma membrane, increased localised wall deposition and some degree of disorientation of the cortical microtubules (Bouck and Galston, 1967). Cells which normally elongate rapidly were no longer capable of this enlargement and thus wall deposition proceeded in the absence of longitudinal strain; the wall microfibrils being deposited with random orientation. In untreated cells the microtubules usually lay in regular patterns parallel to the wall microfibrils.

The involvement of strain on wall microfibril orientation has been recently investigated in *Nitella* (Gertel and Green, 1977). When all strain is suppressed, physically rather than chemically as above, microfibrils at the innermost portion of the wall showed much less than the normal degree of parallelism. This suggests that in the absence of strain in this case, something like a secondary wall (which in *Nitella* has a random microfibril orientation) is deposited. Promotion of transverse strain while longitudinal strain was suppressed or promotion of longitudinal strain during suppression of transverse strain did not alter the orientation of newly-deposited microfibrils. If this latter treatment was carried out in the presence of colchicine, transversely oriented microfibrils were again deposited when the
cells were returned to normal growth medium in the absence of any externally imposed strains.

1.4.4 Secondary Walls

A year after the first description of cortical microtubules adjacent to primary walls in elongating root tip cells (Ledbetter and Porter, 1963), similarly situated microtubules were seen in association with the secondary wall thickenings in developing xylem elements (Hepler and Newcomb, 1964; Wooding and Northcote, 1964). The alignment of the microtubules paralleled that of the wall microfibrils in the developing thickenings. This parallelism has now been observed in many cell and tissue types, adding further evidence for the involvement of cortical microtubules in ordered wall deposition.

*Developing Xylem Elements:*

The alignment of the cellulose microfibrils parallel to the axis of the bands of secondary wall thickenings in developing xylem elements has been well-documented (Roelofsen, 1959). The relationship between microtubules and developing xylem thickenings has been widely confirmed since its initial description, as a result of studies of xylem differentiation in a range of tissue types and circumstances. These include: *Avena* (Cronshaw and Bouck, 1965) and *Triticum* coleoptiles
(Pickett-Heaps, 1966, 1967a, 1968; Pickett-Heaps and Northcote, 1966a); Cucumis (Goosen-de Roo, 1973a, b) and Pinus hypocotyls (Itoh, 1976a, b); leaf veins (Esau et al., 1966; Srivastava and Singh, 1972a); root tips of Triticum (Pickett-Heaps and Northcote, 1966a; Pickett-Heaps, 1966, 1967a, 1968); Brower and Hepler, 1976), Allium (Brower and Hepler, 1976), Medicago (Maitre and De, 1971); secondary xylem development in Acer (Wooding and Northcote, 1964); and Salix (Robards, 1968); the differentiation of parenchyma cells following wounding in Coleus (Hepler and Newcomb, 1964; Roberts and Baba, 1968; Hepler et al., 1970; Hepler and Fosket, 1971) and in Nicotiana pith cultures (Cronshaw, 1967). All these reports confirm the presence of microtubules overlying the thickenings once they have begun to form but only Pickett-Heaps (1966, 1974) has described their role in early stages of differentiation. Initially the transversely oriented microtubules are distributed relatively evenly along the side walls of the xylem cells. The next stage is characterised by a grouping of microtubules into regularly spaced bands, before any thickening formation is evident. Secondary wall deposition then commences underneath the groups of microtubules.

Treatment of cells at early stages of wall deposition with colchicine leads to the loss of the majority of microtubules and irregularly shaped and distributed wall thickenings are formed. In cells at later stages of development at the time of
colchicine treatment, the secondary wall material is often spread out over the wall, covering 2 or 3 thickenings (Pickett-Heaps, 1967b; Hepler and Fosket, 1971). A similar loss of patterning of secondary bands also results following treatment of *Allium* and *Triticum* root tips with the drug isopropyl-N-phenylcarbamate [IPC] (Brower and Hepler, 1976).

Microtubules are thus important in the determination and maintenance of the banded pattern of secondary wall in xylem elements. Other factors may also be involved. In *Coleus*, even in the presence of colchicine, secondary thickenings of adjacent elements are deposited directly opposite each other across the intervening primary wall (Hepler and Fosket, 1971).

**Developing Fibres and Sieve Elements:**

In gelatinous fibres which produce a thick wall of highly pure and crystalline cellulose (Robards, 1969) and in cotton fibres in the zone of secondary wall formation (Westafer and Brown, 1976; Willison and Brown, 1977) the microfibrils and cortical microtubules are both aligned parallel to the long axis of the cell. During wall formation in fibres in the secondary xylem of *Salix*, microtubules consistently paralleled the microfibril orientation at all stages of development (Robards and Kidwai, 1972). This situation existed even though the fibres were synthesising wall microfibrils
with more than one orientation. Bridges between the microtubules and the plasma membrane appeared to result in a tighter attachment of the membrane to the gelatinous wall layer. Another case of localised wall deposition occurs in the formation of bordered pits. In *Salix*, the microfibrils are arranged in a circular or tangential fashion within the border (Robards and Humpherson, 1967). The microtubules appear to be also arranged in a circle around the lip of the pit border.

A difference in the distribution of microtubules in the peripheral cytoplasm of developing fibres has been observed in comparison of normal apple wood with that affected with "rubbery wood" disease (Nelmes *et al.*, 1973). In diseased wood, lignification of the fibre wall is not complete, the wall texture is not as compact and the wall is abnormally thick. It was suggested that microtubules may be concerned with channelling wall substances which are essential for certain steps in polymerisation of the amorphous component of the wall.

Cortical microtubules are present during wall thickening in sieve elements of pine wood (Srivastava and O'Brien, 1966b) and in phloem cells in wheat coleoptiles (Pickett-Heaps and Northcote, 1966a). In pine, the thickened sieve cell wall is a cross-helical polylamellate structure in which the predominant microfibril orientation is greater than 45° with respect to the cell axis (Chafe and Doohan,
1974). However, in *Cucurbita* petioles the lamellae in the sieve tube walls are composed of transversely aligned microfibrils (Deshpande, 1976c). Observation of microtubules in these latter cells was not reported (Deshpande, 1976c) nor was their orientation in pine sieve elements given (Srivastava and O'Brien, 1966b). Microtubules in the phloem cells in wheat coleoptiles did not possess any obvious organisation (Pickett-Heaps and Northcote, 1966a).

*Other Cells with Polylamellate Walls:*

A direct correlation between microtubule alignment and secondary wall microfibril alignment is not always immediately apparent in collenchyma cells of *Apium* (Chafe and Wardrop, 1970). As in *Oocystis* (Robinson *et al.*, 1976; Sachs *et al.*, 1976) the situation is complicated by the repeated changes in the direction of microfibril deposition. In the collenchyma cells, the secondary walls consist of a series of lamellae which are alternately arranged parallel to and perpendicular to the cell axis (Chafe and Wardrop, 1970; Deshpande, 1976a,b). Occasionally lamellae with an intermediate orientation are evident. Just as in *Oocystis*, in many instances the cortical microtubules parallel the most recently deposited microtubules, but in other cases the microtubules are oriented perpendicular, or at an intermediate angle, to the innermost microfibrils. Lack of parallel alignment between the microtubules and the inner microfibrils may indicate that the microtubules exhibit a preparatory alignment, ready to influence the orientation of the
microfibrils of the next lamella (Chafe and Wardrop, 1970). Similar lamellation is described in the tangential walls of epidermal cells in petioles of *Apium*, *Eryngium*, *Rumex* and *Abutilion* as well as that of *Avena* coleoptiles (Chafe and Wardrop, 1972) and in the epidermal walls of lettuce hypocotyls (Sawhney and Srivastava, 1975). Along the radial walls in the first five of these plants, the microfibrils are transversely oriented, the lamellae being continuous with the transverse lamellae in the tangential walls. Details of microtubule disposition are given only in the lettuce epidermal cells where the microtubules are parallel to the inner wall microfibrils. In bean hypocotyl cells microtubules are also transversely oriented along the radial walls but are randomly oriented along the tangential walls (Shibaoka, 1974).

**Seed Coat Palisade Cells:**

In the seed coat of lima beans the orientation of the cellulose microfibrils in the longitudinal walls has been determined using X-ray diffraction patterns (Sterling, 1975). The microfibrils were arranged with their long axis parallel to the long axis of the palisade cells. However, no systems of microtubules were found at any stage of wall development in this study and in view of the orderly arrangement of cellulose the author viewed the absence of microtubules during cell wall thickening to be a "powerful argument against their
supposed participation in the deposition of cellulose microfibrils". The tissue was, however, fixed in ice-cold glutaraldehyde. It would be more instructive if tissues fixed in non-chilled glutaraldehyde were also examined.

**Developing Guard Cells:**

As a final example, the correlation between microtubule orientation and cellulose microfibril orientation during the development of the thickened wall surrounding the stoma of guard cells will be considered. Before any wall thickening is evident in the young guard cells, microtubules collect in a discrete zone adjacent to the plasma membrane along the common wall between the guard cells (Srivastava and Singh, 1972a; Singh and Srivastava, 1973; Hepler and Palevitz, 1974; Palevitz and Hepler, 1976). The microtubules fan out from this zone, which is the future pore site, towards the other sides of the cell. New cellulose microfibrils are deposited in the wall adjacent to the microtubules and oriented parallel to them. Polarized light microscopy showed that the guard cells gradually assume a birefringence pattern characteristic of microfibrils radiating from the pore site (Palevitz and Hepler, 1976). Both microtubule and microfibril orientation remains constant during pore development. Treatment with colchicine results in the loss of the microtubules
and recently deposited wall material is abnormally distributed or "smeared"; the normal birefringence pattern is also absent.

1.5  **Pre-prophase Arrays of Microtubules**

In their report in 1963, Ledbetter and Porter noted that the cortical microtubules which are distributed adjacent to the cell walls during interphase, are no longer present once the mitotic spindle had formed. However, it was not until the studies of Pickett-Heaps and Northcote in 1966 (1966b, 1966c) that the existence of a distinct, intermediate microtubule array was realized. Before cells enter prophase a band, sometimes consisting of 150 or more microtubules, encircles the nucleus at the site of fusion of the future cell plate with the parental walls. The band spreads along the wall for a distance of 2-3 µm, the microtubules often 3 or 4 units deep. Such "pre-prophase bands" were seen in the meristematic cells of wheat roots and coleoptiles (Pickett-Heaps and Northcote, 1966b) and during both symmetrical and asymmetrical divisions leading to the formation of stomatal complexes in young leaves (Pickett-Heaps and Northcote, 1966c).

Since this discovery, many observations of pre-prophase arrays have been made in a wide range of plant tissues, but it would seem that their requirement is not absolute as there is a number of situations in which cell division is not preceded by the formation of a pre-prophase band. A full discussion of the presence or absence of pre-prophase bands prior to division in different tissue and
Speculation as to the exact function of the pre-prophase band range from proposals of an active role in positioning the nucleus or the spindle (Burgess and Northcote, 1967; Burgess, 1970b) to their being, not a causal factor in cell division, but a result of a high concentration of tubulin following breakdown of the interphase wall microtubules prior to formation of the spindle (Pickett-Heaps, 1974; Hepler and Palevitz, 1974; Hepler, 1976). The possible function(s) of the pre-prophase array of microtubules will also be discussed in greater detail in Chapter 5.

1.6 CONCLUDING DISCUSSION

1.6.1 Cortical Microtubule Function

The literature gives much circumstantial evidence for microtubule involvement in the orientation of the microfibrillar component of plant cell walls. In the majority of studies, both of primary and secondary wall formation, deposition of non-random wall microfibrils occurs in the presence of arrays of microtubules lying in the cortical cytoplasm of the cell. The orientation of the microtubules in the arrays mirrors that of the newly-deposited microfibrils in the walls. But by what mechanism do microtubules influence the orientation of wall microfibrils?

Early proposals of cellulose synthesis inside the microtubules (Wooding and Northcote,
1964; Marx-Figini and Schulz, 1966) received little support and were later refuted (Marx-Figini, 1971). More support has been given to the possible role of microtubules in the orientation of cellulose synthetase enzymes or enzyme complexes in or on the plasma membrane. Synthesis and orientation of cellulose microfibrils by enzyme complexes located on the plasma membrane was suggested by Preston in 1964 but he did not invoke participation of microtubules in the alignment of the complexes. Microfibril orientation was envisaged as being achieved by spatially-ordered enzyme complex granules near the cell surface. Particles between the plasmalemma and the cell wall were observed in thin sections in differentiating xylem cells in Salix (Robards, 1969) and particles were also seen on the surface of the plasmalemma in a number of cell types in higher plants using freeze-etching techniques (Chafe and Wardrop, 1970; Willison and Cocking, 1972, 1975) but their distribution bore no correlation to either the wall microfibril or to the cortical microtubule orientations.

In 1974 two mechanisms by which wall microfibrils could be aligned by cortical microtubules were proposed (Heath, 1974a; Hepler and Palevitz, 1974). In one hypothesis (Heath, 1974a), directed movement of cellulose synthetase enzyme complexes in the plasmalemma was envisaged as being produced by a component (possibly the cross-bridges between the
microtubules and the plasma membrane) which inter­acted with both the synthetase complexes and the cytoplasmic microtubules to generate a sliding force, utilising the microtubule as a rigid guiding track. The suggestion of Hepler and Palevitz (1974) was that the cross-bridges generate an oriented flow of fluid components within the membrane, resulting in the alignment of macro-molecular components, including the cellulose synthesising enzymes. Either the microtubule-membrane bridges could actively cause the flow, or the directed movement caused by the sliding of adjacent microtubules relative to one another could be transmitted through the cross-bridges.

In the last two years, using freeze-fracturing techniques, membrane particle arrays which correlate with the orientation of the wall microfibrils have been observed associated with the plasma membrane of higher plant cells. These observations have followed the finding that the treatment of tissues with glycerol prior to freezing resulted in gross plasmolysis of the cells which could cause modifications of the structure of the plasmalemma (Willison, 1975, 1976). Rows of particles associated with the plasma membrane have been seen in Phaseolus root tip cells (Willison, 1976), in Pelvetia egg cells shortly after fertilisation (Peng and Jaffe, 1976) and also during wall formation in the alga, Oocystis (Brown and Montezinos, 1976; Montezinos and Brown, 1976; Brown and Willison, 1977).
Granules associated with the plasma membrane and thought to be cellulose microfibril synthesising centres have also been seen in stelar tissues in corn roots (Mueller et al., 1976), in developing cotton fibres (Willison and Brown, 1977), in regenerating protoplasts (Robenek and Peveling, 1975) and also during cellulose synthesis by the bacterium, *Acetobacter xylinum* (Brown et al., 1976). Similar granules have been described in association with chitin synthesis (Ruiz-Herrera et al., 1975; Bracker et al., 1976).

Thus, current investigations are revealing the association of cellulose microfibrils with granule complexes in or on the plasma membrane in a wide variety of tissues. In some cases it has been possible to show that the organisation of the complexes corresponds to the alignment of the cellulose microfibrils. As yet, no one study has shown that the alignment of the granules matches that of the cortical microtubules underlying the plasma membrane. However, this may be inferred by combining the above reports with information gained in other studies. The parallel alignment of wall microfibrils and cortical microtubules along the longitudinal walls in root tip cells is well documented and appears to be general (Ledbetter and Porter, 1963; Ledbetter, 1965; Northcote and Lewis, 1968; Brennan, 1970). Hence, the parallel alignment of cortical microtubules and particle arrays in the plasma membrane may be
deduced at least for the root tip cells of *Phanerolus* [studied using freeze-fracturing by Willison (1976)]. Similarly, other studies have shown the parallel alignment of cortical microtubules and wall microfibrils in *Oocystis* (Robinson *et al.*, 1976; Sachs *et al.*, 1976) and again it may be deduced from the results of Brown and co-workers (Brown and Montezinos, 1976; Montezinos and Brown, 1976; Brown and Willison, 1977) that this same alignment is shared by the membrane-bound complexes. Clearly there is a need for integrated studies of microfibrils, microtubules and particle arrays, using the same material. Further progress in determining the mechanism by which the cell mediates its morphogenesis by means of microtubules must await such studies. Meanwhile, the purpose of the work reported in this thesis is to provide basic information on the geometry and the development of cortical microtubule arrays.

1.6.2 Cortical Microtubule Organisation

Two often quoted "exceptions" to the parallelism between the wall microfibrils and the cortical microtubules are the primary wall formation in radish root hairs (Newcomb and Bonnett, 1965) and the polylamellate secondary wall formation in collenchyma cells (Chafe and Wardrop, 1970). In both, the discrepancies can be accounted for if the organisation of the microtubule arrays precedes ordered wall deposition. The formation of the
helical microtubule array in the tail of
Poteriochromonas (Schnepf et al., 1975) precedes
deposition of the helically arranged microfibrils
of the lorica. The situation in Oocystis (Sachs
et al., 1976) can serve as a direct comparison to
that in the collenchyma cells. Again, the micro-
tubule arrays are oriented parallel to either of
the two lamellae directions. The grouping of micro-
tubules along the wall of a young xylem element,
and at the future pore site in a developing guard
cell, are additional examples of the organisation
of microtubule arrays in advance of the orderly
deposition of wall microfibrils.

The prediction of the pattern of future wall
deposition by interphase cortical microtubule arrays,
or that of the future site of fusion of the cell plate
by pre-prophase bands of microtubules, emphasises the
crucial need to determine the manner in which the
microtubule arrays are themselves set up and organised.
MTOCs have been identified for the cytoskeletal
microtubule arrays in Ochromonas (Brown and Bouck,
1973) and Pediastrum (Marchant, 1974a), but no
evidence of similar sites has been observed for the
extensive or localised arrays of cortical microtubules
in higher plant cells. The detection of these
initiating sites must be of high priority in studies
aimed at gaining an understanding of plant cell
morphogenesis.
FIG. 1.2  From Ledbetter and Porter, 1970. Cortical microtubules in plant cells both at interphase (A) and pre-prophase (B) are depicted as complete hoops, encircling the cell.
CHAPTER 2

MORPHOGENESIS IN AZOLLA PINNATA ROOTS

2.1 INTRODUCTION

The origin and development of the form of a mature plant, i.e. the plant morphogenesis, is under basic genetic control but is regulated by both internal and external forces. Cells produced in meristematic areas become differentiated from one another and form the distinct tissues and organs of the mature structure. Unlike animals, plants possess permanent embryonic regions - the terminal shoot and root apices and the lateral vascular and cork cambia. Because plant cells cannot move in relation to one another as can animal cells, development leaves a record of its course in the structure of the growing system itself. Changes of form in plants are the result of differences in the location and orientation of cell divisions, in the size and shape to which individual cells grow and in the modes in which cells differentiate.

Within the plant kingdom there is an enormous range in size, from unicellular algae to giant seed-bearing trees, and a corresponding range of complexity of anatomy and life-cycle. The ferns lie somewhere between these two extremes, both in structure and life-cycle. They share with the seed plants the possession of well-developed conducting tissues, xylem and phloem, but differ from them in lacking
the development of seeds. Internally they are more complex than mosses and liverworts, yet in life-cycle they are very similar.

In many ferns, including the water fern *Azolla*, growth of the plant is governed by the activity of apical cells, one at the apex of the shoot and one at the apex of the root (Bower, 1889). The resulting patterns of development in the root and shoot apices of these plants are less complex than those in the multicellular apical meristems of angiosperms, and it is possible to analyse them more fully.


The *Azolla* root provides an unusual combination of advantages for a study of morphogenesis. It is small enough to make comprehensive examination by electron microscopy feasible, yet it retains most of the complexity of larger meristems. Every file of cells can be identified. The root consists of a helix of structural units, each one
originating as a product of a division at the apical cell. These structural units will be termed merophytes (a term widely used in the literature on Bryophytes, introduced by Douin (1923) to refer to equivalent "parts of the plant"). They can be recognised in all parts of the root, even after extensive cell division and differentiation has taken place within them. The merophytes can be classified into a numbered developmental sequence, along which the events of division and differentiation can be mapped, thus enabling stages of development of practically every cell to be categorised.

This Chapter describes the cell lineages that are found in the root; the types, orientations, and locations of cell divisions; the determination of patterns of cell differentiation; and temporal changes that occur during root development. This essential basic information has then been used and extended in the studies described in the following Chapters (3 to 6): in the determination of the structure of arrays of microtubules found in the cortex of the cells (Chapter 3); in investigation of the patterns of microtubule distribution during formative and proliferative division cycles and during differentiation in selected cell types (Chapter 4); in the analysis of the anticipation of planes of division throughout the root by pre-prophase bands of microtubules (Chapter 5); and in preliminary experimental studies on root development (Chapter 6).
2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Culture of locally collected *Azolla pinnata* R. Brown (see Aston, 1973 for Australian distribution) in Hoagland's solution in a range of light and temperature regimes resulted in poor plant growth but prolific growth of a number of green algae. High salt concentrations in nutrient media have been found to kill *A. pinnata* in three weeks (Le Van and Sobachkin, 1963, ref. in Moore, 1969). These authors found it necessary to dilute the nutrient medium 5- to 10-fold. Dilutions (down to 1/50th) of the Hoagland's solution reduced algal growth but did not sustain good growth of Australian material (Fig. 2.1). It was found that supplementation of the dilute Hoagland's solution with a small amount of soil extract or potting soil, or simply growing the plants in local pond water with a little decaying vegetable matter from the bottom of the pond, supported good growth. The addition of small quantities of organic substances obtained from decomposing vegetable matter to mineral nutrient solutions has been found necessary for good growth and development of a number of aquatic plants including *Lemna minor*, *L. major* (Bottomley, 1920a), *L. trisulca* (Hardham, unpublished data), *Salvinia natans*, *Limnobrium stoloniferum* and, to a lesser extent, *Azolla filiculoides* (Bottomley, 1920b). Plants were thus grown in tanks of pond water although
more recently a nitrogen-free culture medium
(Peters and Mayne, 1974a) has also been used.

The morphology of *Azolla pinnata* plants
was found to be quite variable. In the field the
leaves contain an anthocyanin pigment giving the
colony as a whole the red colour seen in Fig. 1.1.
At lower light intensities the plants lack this
red colouration and appear quite green (Figs. 2.2,
2.3). The culture medium was also found to
influence the leaf pigmentation as well as having
a marked effect on the branching pattern of the
plant. In general, the nitrogen-free medium caused
increased growth of the lateral branches (Fig. 2.6).

As noted by Sud (1934), a more intense red
pigmentation developed when the plants began to
fruit. Even after growing well for some time,
colonies died back and produced sporocarps (Fig. 2.5),
which arise from the ventral lobe of the lowest leaf
of a branch (Campbell, 1893, 1905).

2.2.2 Processing of Plant Material for Light and
Electron Microscopy

Tissues embedded in either glycol
methacrylate (GMA) or in Spurr's resin (Spurr, 1969)
were used for light microscopy. Roots were fixed
for 2-3 hours in either 2.5% glutaraldehyde in
0.025M phosphate buffer (pH 7) or in 2.5% glutaralde-
hyde plus 2% formaldehyde in the same buffer and
then rinsed in buffer for 20-30 minutes. For
embedding in GMA, the roots were dehydrated in methyl cellosolve, ethanol, n-propanol and n-butanol (Feder and O'Brien, 1968), infiltrated with GMA for 1-2 weeks and the GMA polymerized under nitrogen and ultraviolet light. The GMA was purified following the procedure outlined by Tippett and O'Brien (1975).

For embedding in Spurr's resin, roots were post-fixed in 2% OsO₄ in 0.025M phosphate buffer, rinsed and then dehydrated in a graded series of ethanol or acetone solutions. All processes were carried out at room temperature.

Sections were cut on a Reichert OMU3 ultramicrotome. For light microscopy sections 1-2μm in thickness were stained with 0.05-0.5% Toluidine Blue O in sodium tetraborate buffer (pH 9.3) for Spurr's resin-sections or in acetate or benzoate buffer (pH 7.2) for GMA sections. Ultra-thin sections for electron microscopy were stained in a saturated solution of uranyl acetate in 50% ethanol and in lead citrate (Venable and Coggeshall, 1965) and viewed in a JEOL 100B or a Hitachi H500 electron microscope.

Roots were also cleared by brief boiling in lactophenol, mounted on glass slides in fresh lactophenol and viewed in the light microscope using Nomarski interference-contrast optics. The roots were not squashed or flattened, the coverslip being separated from the slide by two strips of "parafilm".
2.3 RESULTS

2.3.1 General Description

Mature roots of *Azolla pinnata* attain a maximum length of approximately 50mm, although elongation may cease before this length is reached (Fig. 2.1). Young roots are enclosed in a root cap (Figs. 2.4, 2.8) which is approximately 5mm in length when mature (Figs. 2.7, 2.8) and reaches a maximum diameter of approximately 0.3mm by the time the root is 10mm in length (Figs. 2.7, 2.8, 2.48). The cap continues to cover the root tip until it is sloughed off when the root has attained its full length (Fig. 2.7). Root hairs emerge from the root cap as cell elongation in the root pulls out their entire 1.2-1.6mm length from within the cap (Figs. 2.7, 2.8).

Transverse and longitudinal light microscope sections (Figs. 2.9-2.18) serve to introduce the cell complement of the mature root, and its layout. The overall radius of the root is 85µm, of which the epidermal layer occupies 15µm, the inner and outer layers of cortical cells 26µm and 24µm respectively, and the stele the remaining 20µm. The epidermal layer contains a somewhat variable number of cells, but 18 is the most usual. The inner and outer cortical layers consist of rings of 6 and 12 cells respectively (Fig. 2.14).

The stele is constant in its construction
and consists of two concentric rings surrounding a central group of cells. The outermost ring or hexagon is formed by 6 endodermal cells. The cell layer lying internal to the endodermis consists of 6 pericycle cells plus 2 sieve elements. The pericycle cells develop wall ingrowths of the transfer cell type on the walls which back onto the xylem elements (Gunning and Patte, 1974), so that in functional terms xylem parenchyma might be a more apt name. Four of the 6 endodermal cells, in two opposite pairs, each have one pericycle cell lying against their inner tangential walls. In each member of the third pair of cells in the pericycle layer, insertion of a radial wall gives rise to sister cells, one of which remains as pericycle, while the other differentiates as a sieve element (described hereafter as the outer sieve element).

The final group of cells in the stele is the innermost hexagon. Internal to each of the pericycle-outer sieve element pairs there is a parenchyma cell, flanked on either side by a xylem element and another sieve element (the inner sieve element). The parenchyma cell does not develop wall ingrowths where it backs onto the xylem, and is designated as phloem parenchyma. It is not a direct sister cell of either sieve element. The roots exist in two mirror image forms (Figs. 2.14,2.15) and the two enantiomorphs will be referred to as right-handed and left-handed. Transverse sections of both forms
show two-fold rotational symmetry about the central axis of the root.

2.3.2 The Apical Cell

Longitudinal and transverse views are shown in Figs. 2.19, 2.23-2.26, 2.29-2.31. The curved distal face (chord approximately 22µm) of the apical cell is in contact with the root cap. The three proximal faces are flatter and approximately triangular, tapering to the apex of the cell (distance from apex to curved face along the root axis averages 10.6µm). Plasmodesmata are abundant in these walls and sparse in the distal wall. The ultrastructure of the apical cell varies according to the length of the root that is being examined. In roots longer than about 0.8mm, the apical cell becomes more and more vacuolated (cf Figs. 2.23, 2.24), though its dimensions do not alter.

2.3.3 Segmentation of the Apical Cell

Earlier authors have described the origin of the root apical cell and its first division (Strasburger, 1873; Campbell, 1893; Leavitt, 1902; Rao, 1935, 1936; Demalsy, 1953, 1958). It is adventitious, derived from a cell which, in parts of the shoot apex other than where roots are being formed, would become partitioned to give rise to elements of both the cortex and the stele of the shoot. The apical cell is tetrahedral. One face
lies in an outer periclinal position, and the first division occurs parallel to that face, cutting off a cell which by one further periclinal division and then numerous anticlinal divisions, generates the whole of the 2-layered root cap. The outermost cell layer of the shoot proliferates to form a sheath of cells outside the root cap (Figs. 2.23, 2.25, 2.26, 2.29-2.31). After its first periclinal division the apical cell enters a new mode of behaviour, described below, that persists for the rest of its active life.

Segmentation of the apical cell has been examined by means of both longitudinal sections and serial transverse sections. Divisions occur parallel to each of the three proximal tapering faces in turn, giving a sequence of overlapping cells, seen in transverse section in Figs. 2.34-2.36 and in longitudinal section in Figs. 2.25, 2.26, 2.29-2.32. The pattern of overlapping seen in transverse section shows whether the divisions follow a right-handed or a left-handed sequence. 50% of the apical cells were right-handed and 50% left-handed; the two types alternating along a frond as shown in Figs. 2.4 and 2.41, there being an invariable correlation between apical cell behaviour and the two mirror image forms of cell distribution in the mature parts of the root.

The consequence of development by sequential divisions at an apical cell is that the plant is
built up of successive segments, or merophytes, each one derived from one apical cell division process. In the Azolla root, three successive divisions generate one complete gyre of a helix of merophytes in which the three neighbours are displaced from one another along the long axis of the root by a distance equal to about one third of the longitudinal dimension of each one. This relationship survives, even though the length of each merophyte may increase up to about 1,500-fold as the root matures (Fig. 2.42). The successive turns of the helix produce three longitudinal files of merophytes each one occupying a 120° sector of the root (Figs. 2.19-2.22). Moving away from the root apex along any one of the three files, the successive merophytes represent developmental increments, each equivalent in duration to three apical cell cycles.

2.3.4 The Formative Divisions within Merophytes

The cell complement of the mature root arises by means of two classes of cell division. First, the initial cells of the cell files are generated by a sequence of formative divisions within each successive merophyte. Enlargement, in both radial and longitudinal directions, accompanies these formative divisions, so that the diameter of the root approximately doubles (Fig. 2.13). Second, the initial cells that are formed by the formative divisions undergo proliferative divisions
in the transverse plane; once again, surface extension accompanies cell division.

Serial transverse sectioning through the zone of formative divisions reveals that this sequence of divisions is very regular. Every cell in 16 root apices was examined by serial sectioning without evidence of substantial differences emerging. Four micrographs of sections through the apical cell and the first 9 merophytes of one root (Figs. 2.33-2.36) illustrate how the cell complement of each merophyte is determined. Examples of sequences of merophyte development are diagrammed in Fig. 2.43 and data on the precision of the sequencing appears in Fig. 2.44. A flow sheet that summarises the cell lineages is shown in Fig. 2.47. Arbitrary code numbers have been assigned to each type of division in the lineages (see Fig. 2.46).

The number of undivided merophytes found adjacent to the three proximal faces of the apical cell before the first division is encountered ranges from one to five according to root age (Figs. 2.19-2.22,2.33,2.34,2.43, Table 2.1). No divisions which would place a cell wall transverse to the long axis of the root have been observed in either transverse or longitudinal views in any of these segments. The first division to occur (division 1, Fig. 2.46) is always tangential-longitudinal, the wall forming closer to the exterior of the root than to the centre (Fig. 2.20). As far as can be seen by microscopic
examination, in this and subsequent stages throughout the formative divisions, no segment reaches a stage in advance of its next older neighbour in the helix (Fig. 2.43). It is true that a pair of adjacent segments can look the same in respect of their internal partitioning, but the older member of the pair could well be at a more advanced stage in its cell cycle. Conversely, for its further development a given merophyte does not have to wait until the next younger member of the helix of merophytes has reached the same stage of development.

In theory, the sister cells produced at division 1 could develop either synchronously (Fig. 2.43A) or asynchronously. However, the outermost cells, which are the precursors of the epidermal and outer cortex cell layers of the root, are usually the first to lay down a new partition (Figs. 2.21, 2.34, 2.43B,C,D). The first division wall in the outermost cells is radial-longitudinal and divides the parent cell, which itself occupies 120° of the circumference of the root, into a 40° and an 80° sector (division 2a, Fig. 2.46). When the helix of segments, whether it be right-handed or left-handed, is followed around the root, there seems to be no consistent location for the new radial-longitudinal wall: in some roots the 40° sector lies before the 80° one; in others the reverse is seen (Figs. 2.34-2.35, 2.43).
A second radial-longitudinal wall, which partitions the 80° sector symmetrically, is then laid down (division 2b), but before this happens it is usual (Fig. 2.43) for the inner cell formed at division 1 to divide radially (division 3) giving two equal 60° halves (sextant cells) in each segment (Figs. 2.22, 2.28, 2.36). These sextant cells ultimately give rise to the inner cortex and the stele (Fig. 2.47).

Tangential-longitudinal divisions then take place in two locations: (1) the outermost 40° sector cells (division 4), and (2) the innermost sextant cells (division 5, Figs. 2.28, 2.43). These two types of division can occur concurrently.

Division 4 gives rise to the dermatogen (the precursor of the epidermal layer) and to the outer cortex. The dermatogen cells usually pass through one round of radial partitioning (division 6, Figs. 2.43, 2.46) and two rounds of division in the transverse plane of the root before the highly asymmetrical division which generates trichoblasts and atrichoblasts (division 10) occurs (Figs. 2.13, 2.18, 2.43, 2.46). One consistent feature of division 4 is that the tangential walls formed in the three 40° sectors of the dermatogen-outer cortex precursor layer are not laid down in precise continuity with one another. In the central 40° sector the wall lies slightly further to the exterior of the root, as compared with the position of the same wall in the two neighbouring 40° sectors (Figs. 2.9-2.11,
2.43. There is then an additional division (4a) in the radial-longitudinal plane in the central 40° sector, completing the formation of the initial cells for the outer cortex (Fig. 2.46) and giving a ring of 12 cells (Fig. 2.14).

Division 5, in the inner sextant cells, can be, but is not always, synchronous in sister cells, i.e. those within a 120° merophyte. It is tangential, and the subsequent development of the outer cell that is formed by it is the same in all merophytes: a further tangential-longitudinal division (division 7) completes two more cell lineages by giving rise to the initial cells for the inner cortex and the endodermis (Figs. 2.9, 2.43). The fate of the inner cell formed at division 5 is, however, not the same in each merophyte. First of all there is another tangential-longitudinal division in every sextant (division 8, Figs. 2.10, 2.11, 2.46) giving totals of 6 inner and 6 outer cells which differentiate in a strictly controlled manner to produce the mature configuration that has already been described. Four of the outer cells formed at division 8 become pericycle initial cells directly. The two remaining outer cells, comprising a pair on opposite sides of the root, divide radially (division 9), one product being the outer sieve element initial cell and the other a pericycle initial cell. The cell internal to each site of division 9 becomes a phloem parenchyma initial cell and the remaining four inner
cells become an opposite pair of xylem initials and an opposite pair of inner sieve element initials, arranged so that the inner and outer sieve elements lie close together, each one sharing a wall with an intervening phloem parenchyma cell (Figs. 2.11-2.12, 2.14-2.17, 2.46).

The disposition of the cells in the two inner hexagons of the stele is fixed in relation to the handed-ness of the apical cell segmentation. Considering the layer immediately within the endodermis, and following the direction of the helix, whether it be right- or left-handed, the outer sieve element always lies before the small pericycle cell that is separated from it by division 9 (Figs. 2.12, 2.14-2.17).

2.3.5 Proliferative Divisions

The initial cells at the mature extremities of the branches in the cell lineage diagram (Fig. 2.47) are continually being produced in young roots, thereby generating files of cells. Further development within the files can be monitored in longitudinal sections, where it is possible not only to identify the boundaries of the successively older merophytes, but also (given practice) to identify the cell types, even though not all are represented in a given section. By mapping the transverse cell walls within files, and noting the position of the proximal and distal transverse boundaries of
each merophyte, the number and site(s) of the transverse proliferative division cycles undergone by each initial cell can be found.

The simplest situation is that of the two metaxylem elements. They appear to be very similar to one another, and they arise directly from their initial cells without any division in the transverse plane. This, and other, information is summarised in Fig. 2.46. The different cell files within a particular category of cell type behave similarly, but contrasting with this consistency within cell layers there is variation between cell types (Fig. 2.46). The endodermis initial cells undergo four complete rounds of division and some, but not all, of the 16 cells so formed per file per merophyte enter a fifth round. Accordingly, the mature root has two endodermal cell files per merophyte, each with between 16 and 32 cells. By contrast, the outer cortex initials pass through four successive cycles. The inner cortex initials divide and each daughter redivides, but whereas in the cell files considered thus far the individual cells formed by transverse divisions appear to be equivalent, this is not necessarily so in the inner cortex. In the majority of cases the cell lying closest to the root apex (at the acroscopic face of the merophyte) redivides, thus giving five cells per file per merophyte (Figs. 2.13, 2.18, 2.46). In examining several hundred merophytes, only one
example was found of a group of five arising by division of a cell other than the most apical.

Within the endodermal layer, the four initial cells of large pericycle files resemble those of the endodermis (although endodermis and pericycle initials are not sister cells, Fig. 2.47). They enter, but probably do not all complete, a fifth round of division. The two outer sieve element initials divide once, and then differentiation proceeds rapidly so that the mature files consist of two cells per merophyte (Figs. 2.13, 2.18). The two inner sieve elements and the two phloem parenchyma initials produce eight cells per file per merophyte by three rounds of division.

The dermatogen layer is unique in interspersing proliferative and formative divisions. Two successive transverse divisions give 4 cells in each of the 6 files per merophyte, whereupon an oblique division (division 10) in each of the four gives 4 trichoblasts alternating with 4 atrichoblasts (Figs. 2.13, 2.18). Each of the latter then passes through two more successive transverse division cycles so that each file in each merophyte starts with a root hair at its apical end, then 4 epidermal cells, this pattern being repeated a total of 4 times. Occasionally there is variation in the number of epidermal cells between adjacent root hairs.

Further data on the transverse divisions within
merophytes, with particular reference to the position along the root where the various rounds of division in the dermatogen, cortex and endodermis are initiated are given in Table 2.2 and Fig. 2.45. From the foregoing it is evident that the various types of initial cell each have their own determinate programme, specifying the number of division cycles that will take place.

The dermatogen tends to be the first of the files to enter a given round of division and the endodermis the last (Fig. 2.45). Initially, the temporal ordering is not as precise as in the case of the formative divisions, but as the dermatogen and the inner cortex cells cease cyclins, so the alternation of outer cortex and endodermis divisions becomes more striking. These tissue-specific differences in timing are maintained while the root primordia develop and age.

Examination of the initiation of a round of division within a merophyte has not disclosed any preferred site(s). When two-celled files begin to divide to make four-celled files, either the proximal or the distal cell can divide first. The same applies to subsequent rounds where proximal, distal, or central cells within a merophyte may enter mitosis first. Synchrony within a merophyte is imperfect, so that in the later rounds the divisions may be spread over at least two successive merophytes along any one of the three 120° sectors.
2.3.6 Developmental Changes

Cell divisions, both formative and proliferative, and events of cell differentiation, do not occur at the same position in roots of different developmental stages. In order to investigate this spatial variation, roots were harvested from successive branch sites along individual fronds (Fig. 2.4). Four features were examined in roots of different stages of development: the rate of division of the apical cell, the positions of the formative and transverse divisions, and certain indicators of cell differentiation.

The Apical Cell:

It had previously been found that mature roots carry about 70 bands of root hairs along each of the three 120° sectors. Taken together with the knowledge that each merophyte gives rise to four tiers of root hairs (Figs. 2.13, 2.18), this suggested that the apical cell divides just over fifty times (70 x 3/4). Longitudinal sectioning of roots of different stages of development, and mapping and counting of merophytes, provided confirmation. Fig. 2.49 gives the number of merophytes counted in roots of different lengths, and shows that the mature number is produced by the time the root is about 1.0 mm in length. Thereafter
the apical cell becomes progressively more vacuolated (Figs. 2.23, 2.24).

The approximate duration of the apical cell cycle was estimated at different stages of root development. The procedure used was to count the number of merophytes in each member of a pair of adjacent roots along a frond, using longitudinal sectioning, or clearing in conjunction with observations by interference-contrast optics, as in Fig. 2.38. The root lengths were also measured so that the time interval between the stages of development of the adjacent roots could be estimated by reference to a graph of growth rate versus root length (Fig. 2.51), prepared by making successive length measurements on numerous growing roots covering a wide range of developmental stages (Fig. 2.50). The time interval, divided by the change in the number of merophytes, gave the average apical cell cycle duration for the relevant phase of growth (Fig. 2.53). The duration falls during early stages of root development to a minimum of 3-5 hours.

The Formative Divisions:

The addition of one new merophyte to a root indicates the completion of one more apical cell cycle. Further, the number of merophytes that is needed to attain a given stage in the formative division sequence measures the time, in apical cell
cycles, that has elapsed since inception of the merophyte that has reached that stage. Table 2.1 (left-hand side) shows how these total elapsed times vary with the age of the root. The time intervals between successive stages in the division sequences can also be measured in terms of apical cell cycles (Table 2.1, right-hand side).

Fig. 2.43A depicts the division sequence in a theoretical situation in which the cell cycle duration for every cell matches that of the apical cell. Only 8 merophytes are required to complete the lineages. Real roots, by contrast (Figs. 2.43B, C,D), require 2-3 times more merophytes, indicating that, on average, the cycle durations for the formative divisions are 2-3 times longer than for the apical cell. Reference to Table 2.1 (right) shows that this average conceals non-uniformity. For instance, of the lineage divisions 2a, 2b, 4 and 6, which generate the dermatogen, the first three are each equivalent to 2-3 apical cell cycles while the last is equivalent to 5 apical cell cycles. None of these divisions alters greatly in duration (measured as apical cell cycles) as the root ages. The same applies to division 3 (formation of sextant cells) and division 9 (formation of the outer sieve element).

Other divisions are not geared to the apical cell divisions. Divisions 1 and 5 speed up relative to the apical cell as the root ages,
taking about half as many merophytes for completion in the oldest root that was examined as compared with the youngest. Division 8 shows the same effect but more obviously, and division 7, which separates the endodermis from the inner cortex, displays the most pronounced acceleration (4-5-fold relative to the apical cell) of all of the formative divisions. Fig. 2.44, which shows that the formative divisions follow a set sequence in the majority of roots, includes data from roots of widely different ages. The scattering of some of the points arises from the differential effects of aging on the duration of the cell cycles. Thus division 6 occurs before division 9 in young roots, but the sequence can be reversed in old roots because division 7 (which precedes 9 in the lineage for the stele) proceeds faster.

\textit{Proliferative Divisions:}

The positions of the transverse divisions were mapped by examining longitudinal sections. In order to avoid the practical difficulty of identifying every cell type by serial sectioning (as was done when obtaining data on transverse divisions in stelar cell files, Table 2.2) attention was focused on those cell types which are present in every median longitudinal section, namely the epidermal layer, the outer and inner cortex, and the endodermis.
The results are summarised in Fig. 2.53, where the distance from the apical cell, expressed in terms of the number of merophytes at which various events first happen, is graphed against root length. The main conclusions are as follows:

(i) Transverse divisions do not invade the zone of formative divisions. This applies to the internal cell files but there is evidence (see later) that root hair formation is exceptional in being able to progress further towards the apex (Fig. 2.18).

(ii) The site at which each type of transverse division commences shifts acropetally as the root ages. In some cases (the first two rounds of division in the dermatogen and the outer and inner cortex, Fig. 2.53A,C,D), the shift levels off at approximately the position where the formative divisions have been completed. The levelling off is not apparent in the other cases examined (Fig. 2.53B,D), but probably would have been if older, longer roots had been examined.

By combining data from Table 2.1 and Fig. 2.53, it is possible to estimate the duration of cell cycles: (a) between the formation of an initial and its first transverse division, and (b) between the 1st and 2nd transverse divisions, and also to see how these cell cycles alter as the root ages. There are differences between cell types:
the dermatogen and outer and inner cortex initials have a shorter cycle than the endodermal initials. The second cycle (under (b) above) takes approximately as long as the first (under (a) above) in all cell files except the dermatogen, where the second is twice as long as the first in young roots. There is a pronounced aging effect in the dermatogen and outer cortex (shortening of the duration of the first cycle in older roots) but not in the inner cortex and endodermis. The latter effect might be expected to alter the sequence of divisions (Fig. 2.45), but the age-induced differentials between the inner cortex and endodermis on the one hand and the dermatogen and outer cortex on the other are largely cancelled out by differentials that act in the opposite direction in the formative zone, where the surface layers do not change with age, while division 7, which generates the inner cortex and endodermal initials, accelerates.

(iii) The oblique division that delimits trichoblasts from atrichoblasts (division 10) progresses acropetally from cell to cell along the dermatogen. Only one exception was found in an old root. Apart from that one case, the next cell to undergo this division was always adjacent (apical) to the one that had just divided, using up the merophyte-by-merophyte sets of four dermatogen cells. By relating sites of division to growth data the rate of
progress of the root hair forming division was estimated to be one cell in 100 minutes (mean of 9 determinations). In late stages of root development the division overtakes and precedes the proliferative divisions of the dermatogen initials into two and then four cells per file per merophyte. This results in merophytes near the apex of the root with less than 4 pairs of trichoblasts and atrichoblasts (Fig. 2.18).

(iv) The transverse divisions, as mentioned above, are not confined to a single meristematic zone. Instead, the sites of successive rounds in any given cell file are separated by merophytes in which cell enlargement (mainly elongation) occurs. However, as root development proceeds, the sites of the successive divisions, by their acropetal shift, draw closer together (Fig. 2.53) and eventually the successive rounds can be initiated in the same merophyte (see overlap of points in Figs. 2.53A,B,C). Only a few examples of this were found in the series of roots examined, but it is likely to be general in old roots after apical cell and formative zone divisions have ceased.

(v) The extent of merophyte elongation is greatest at the base of the root (Fig. 2.42), being progressively less in merophytes closer to the root.
apex. Roots were examined to see if the elongation and transverse division processes are related. Fig. 2.54 presents representative data on the merophyte lengths at which division events occur, complementing Fig. 2.53 in which the merophyte numbers for the same events are plotted. Before considering the results the source of major error should be considered. The merophyte that was measured was the one showing the first sign of the given class of division. There was therefore no possibility of accidental underestimation, but over-estimation was unavoidable. It was a matter of chance whether the root was fixed when the first sign of division consisted merely of one cell entering mitosis in the file/merophyte being considered, up to the opposite extreme in which all cells in the file/merophyte had just completed their division. Because root elongation was occurring throughout, there was considerable spread between the above two extremes in the measures obtained for the merophyte length at which a given transverse division was initiated. The chance of obtaining the earliest stage of division was slight, especially in later rounds of transverse division, and the faster the root was elongating (i.e. the older it was, Figs. 2.50, 2.51) the more likely it was that the length that was measured overestimated the length at which the division commenced. This may account for the upward trend of the points in
Fig. 2.54.

Bearing in mind that the major error is biased towards overestimation, the results show that the merophyte length at which a given category of transverse division occurs is remarkably constant in roots of different stages of development. No marked acropetal drifts of the sort seen in Fig. 2.53 are seen, except to a slight extent in the case of the epidermal layer, where the oblique division that delimits trichoblasts from atrichoblasts, and the first round of divisions in the atrichoblasts, both tend to occur in slightly shorter merophytes as root development proceeds. The fourth round of transverse division in the endodermis and the outer cortex occurs when the merophyte has elongated to become twice as long as it was when the previous round occurred. The second rounds in the endodermis, both layers of cortex, and the dermatogen, occur considerably before elongation has doubled the merophyte length with respect to the length at the first round. The same applies to the third round in the outer cortex and endodermis (Fig. 2.54).

(vi) The organisation of the dermatogen lends itself to the detection of abnormalities that are of interest in relation to elongation and cell division. Deviations from the normal pattern of production of four trichoblasts and four intervening atrichoblasts, each of which gives rise to four
epidermal cells, do sometimes occur. Several merophytes were found in which a dermatogen initial cell had divided once transversely, but then only one of the two products had divided again, giving a file of three cells in the merophyte, two being of normal length and the third double-sized. The normal relative proportions of the daughter cells were retained when the large cell divided obliquely giving rise to an unusually large paired trichoblast and atrichoblast. The atrichoblast entered its first transverse division precociously, 6 merophytes closer to the root apex than its normal-sized neighbours. In terms of cell length, however, the double-sized atrichoblasts behaved normally. They entered their first transverse division at the same cell length as did their normal counterparts six merophytes further back along the root. Evidently, their progress towards this division was accelerated by virtue of their greater initial length.

Differentiation:

The acropetal drift of the sites of transverse divisions is paralleled by a similar drift of sieve and xylem element differentiation. Fig. 4.16 shows the merophyte number in which xylem wall thickenings were first apparent (in the light microscope). The progress of this site towards the apex parallels that of the root hair-forming division.
with xylem differentiation slightly in advance. Sieve and xylem element differentiation will be described more fully in Chapter 4.

2.3.7 Abnormalities of Cell Division and Differentiation

Three of the 16 roots which were analysed by serial transverse sectioning possessed a number of abnormalities (Fig. 2.55). In merophyte number 10 in the root diagrammed in Fig. 2.55A, division 3, dividing the central 120° sector into two sextant cells, has not occurred. Divisions 5 and 7 have nevertheless been completed, resulting in tangential-longitudinal walls twice as long as normal. They have still fused with the parent walls in the normal positions. In the second root (Fig. 2.55B) merophytes 11 and 12 both contain abnormalities. In merophyte 11, the central dermatogen-outer cortex precursor cell has completed a radial-longitudinal division instead of the normal tangential-longitudinal division. In merophyte 12, the phloem parenchyma cell has completed an extra, radial-longitudinal division. In merophyte 15 of the third root (Figs. 2.37, 2.55C) the phloem parenchyma cell has completed an oblique, longitudinal extra division.

One colony of plants growing in the nitrogen-free culture medium (Peters and Mayne, 1974a) were found to have a high proportion of their roots possessing abnormalities of xylem differentiation.
In many roots there were discontinuities in the two files of differentiated xylem elements (Fig. 2.38) and in other roots, isolated xylem elements had also differentiated in a third cell of the inner hexagon (Figs. 2.39, 2.40). These observations were made in cleared roots using interference-contrast optics and it was not possible to ascertain if this third cell would have been a phloem parenchyma cell or an inner sieve element.

2.4 DISCUSSION

2.4.1 The Study of Plant Morphogenesis

The morphogenesis of any multicellular plant structure is an interplay of spatial and temporal controls of cell division, determining the sites and numbers of cells, and control of enlargement and differentiation, determining the sizes, shapes and types of cells that are present. The regulation of these controls is based on the genetic program, unique to each species, but involves the interaction of intrinsic and external influences. The morphogenetically active parts of higher plants are the meristems at the root and shoot apices and the lateral vascular and cork cambia. Just as the terminal meristems are more amenable to experimental study than the often inaccessible lateral cambia, the root apex, being less complex than the shoot, offers some advantages over the
shoot apex for investigations into the basic principles underlying apical organisation and morphogenesis.

Basically, the same tissue systems and cell types are formed in the root as in the stem but, unlike the shoot which produces leaves very close to the apical dome, lateral roots, if present, originate at some distance from the root apex and their initiation does not complicate the structure of the apex itself. When median longitudinal sections of roots with multicellular meristems are examined, files of mature and differentiating cells may be traced acropetally into a small number of meristematic layers adjacent to the root cap. The rows of root cap cells may also be traced into this region. The quiescent centre in the root apex was initially described as a result of a geometrical analysis of these cell lineages in the root meristem of Zea (Clowes, 1954).

Two experimental methods which have contributed to the study of morphogenesis in plant roots have been surgical methods by which portions of the root apex are damaged or removed and the resulting development changes studied (e.g. Clowes, 1972; Steeves and Sussex, 1972; Feldman, 1976) and experiments in which tissue (Feldman and Torrey, 1976) or organ cultures (e.g. Feldman and Torrey, 1975; Scadeng and MacLeod, 1976) are grown in sterile nutrient media of controlled composition.
Often labelling experiments using $^3$H-thymidine are carried out independently or in conjunction with these two approaches yielding estimates of cell cycle times in the various tissues of the root apex (Barlow, 1973; De La Torre and Clowes, 1974; Clowes, 1975a; MacLeod, 1976a). Ultrastructural approaches have been very productive in studies of cellular and sub-cellular aspects of differentiation and morphogenesis of selected cell types and relatively simple organisms such as algae. These materials meet the constraint that is placed upon specimen size in transmission electron microscopy, and at the same time they provide useful models for the events that are believed to take place in the larger and more complicated organs of higher plants. These simple models or restricted sub-systems, however, lack the higher levels of morphogenetic complexity that are present in the organs of higher plants, particularly with respect to tissue and cellular interactions.

Root meristems exhibit a wide variety of cell patterns, however, the simplest to analyse are those in roots which possess a single apical cell as is the case in many ferns, including the water fern *Azolla*. The root of *Azolla* is small and amenable to processing for electron microscopy. It provides a stage-by-stage record of its development in the form of a sequence of merophytes that reveals the past meristematic activity of the apical
cell and the way in which the other types of cell division are controlled in time and space. These advantages have permitted compilation of a considerably more detailed portrait of certain cellular aspects of morphogenesis in and near a meristem than was formerly available. This has provided a foundation on which the studies described in Chapters 3 to 6 have been based and upon which investigations may build, leading to a better understanding of morphogenetic processes in plants.

2.4.2 The Apical Cell: Meristematic or Quiescent?

Initial studies of roots with single apical cells (e.g. Azolla, Strasburger, 1873) viewed the apical cell as the initiator of all the tissues of the root, i.e. a single cell equivalent to the multicellular meristematic zone in angiosperm roots. However, more recently there has been controversy about the role of apical cells (Hallet, 1972; Sossountzov, 1972, 1975), with some authors claiming that they are quiescent, most of the meristematic activity being on their proximal flanks. This claim was based upon the fact that the apical cell in Equisetum (Buvat and Laird, 1953; D'Amato and Avanzi, 1968), Marsilia (D'Amato and Avanzi, 1965) and Blechnum and Ceratopteris (Avanzi and D'Amato, 1967) was rarely seen in the process of division and because it was larger and more differentiated in appearance than the surrounding cells. The apical
cell was thought to behave as a typical initial only in the origin and the early organisation of the root primordium (D'Amato and Avanzi, 1965, 1968; Avanzi and D'Amato, 1967). The root apical structure of these ferns was later interpreted as showing that the apex contained a multicellular quiescent centre whose size was dependent on the developmental stage and size of the root apex (Avanzi and D'Amato, 1970; D'Amato, 1975). According to these workers, this quiescent centre started to form when the root initial cell, having formed the primordium, had stopped dividing and with further development other cells adjoining the apical cell also became quiescent. Meristematic activity in the root was attributed to cells on the periphery of this continually enlarging centre.

Measurements of the incorporation of \(^{3}\text{H}\)-thymidine in roots of *Ceratopteris* (Gifford, 1960), indicating that DNA synthesis was taking place in the apical cell, were not considered by the advocates of a quiescent role for the apical cell. In addition, the infrequency of observed division in the apical cell is an unreliable criterion on which to base claims of quiescence. In *Ceratopteris* roots the majority of apical cells were found to divide at a specific period of the day - between midnight and early morning (Chiang, 1972) although a similar limited period of division in the *Azolla* roots was not found in the present work. The
importance of the stage of development of the root when determining the role of the apical cell, is evident from the present study of Azolla pinnata roots and has also been emphasised by Sossountzov (1975) from studies of shoot apices in Equisetum. It is easy to see how an impression of quiescence can be gained if roots of determinate growth habit (like those in Azolla) are examined after cessation of apical cell division activity. The present estimates of apical cell cycle durations for young Azolla roots are similar to the values obtained for young Ceratopteris and other roots by Chiang and Gifford (1971), Chiang (1972) and Chiang and Lu (1972), which are not considered by D'Amato (1975). In addition the pattern of overlap of the successive inter-merophyte cell walls in Azolla is quite inconsistent with the occurrence of transverse division activity in the flanking cells (see also Lintilhac and Green, 1976). Every division that has been seen in these cells has been longitudinal. The divisions that occur proximal to an apical cell that has ceased to cycle are not equivalent to divisions of new initial cells flanking a newly-established quiescent apical zone (cf D'Amato, 1975; Torrey and Feldman, 1977) but rather are merely the completion of the normal sequence of formative and proliferative divisions that occurs even when the apical cell is active. It is not excluded that some endopolyploidy may occur in old apical cells, but
the data on this point are highly variable (D'Amato and Avanzi, 1965), and these authors admit the Feulgen reaction on which their results are based gives atypical staining (Avanzi and D'Amato, 1970).

2.4.3 Cell Generations and Cycle Times

Reference to Fig. 2.47 shows that the maximum number of nuclear generations within a merophyte is found in the epidermal lineage, where the cells are the products of 10-11 successive divisions. If the maximum number of apical cell divisions is about 55 (Fig. 2.49), and if the last 10 or so merophytes remain undifferentiated at the apex of old roots (Fig. 2.18), then the maximum total number of nuclear generations anywhere in the root is in the epidermis of the last merophyte (approximately the 45th) to complete its epidermal lineage. Here the cells represent the products of about 55 cycles (neglecting any endopolyploidy that may occur), commencing at the time the newly de-lineated adventitious apical cell begins to cycle at one or other of its proximal faces. There are no comparable data for other plant materials but these results indicate that the number of successive cell generations may be limited, as claimed for human embryo cells in culture (Hayflick, 1974). The cell populations in these cultures double about fifty times. A limit in the number of cell generations could be the cause of the determinancy of root growth
exhibited by *Azolla* and other ferns.

The greatest difference between a fern root and an angiosperm root is that the latter possess a quiescent centre. The quiescent centre has been shown to act as a reservoir of cells which are relatively immune to perturbations which damage more rapidly cycling cells (Clowes, 1975b). After severe irradiation, the normally rapidly cycling cells stop cycling. The root recovers its growth by the repopulation of the meristem with cells derived from the products of increased meristematic activity within the quiescent centre. The same kind of response occurs after surgical treatments which damage the meristematic cells of the root (Clowes, 1972). Recently, Clowes (1975a) has shown that there is a gradient of cycling time in the quiescent centre, the peripheral cells cycling faster than those toward the centre. By acting as a very slowly cycling reservoir of replacement initials (Clowes, 1976; Barlow, 1976), each capable, like an apical cell, of contributing an alloted quota of cell generations, the quiescent centre could effectively overcome determinacy imposed by limitation of generations.

It is likely that this limitation of generations is also the reason why the last 10 or so merophytes do not complete their formative divisions, resulting in a zone of undifferentiated cells at the root apex. It may also be why the
acropetal progression of division 10, the last division in the epidermal lineage, overtakes the second and then the first proliferative divisions, and even the formative divisions 4 and 1. The over-riding of these formative and proliferative divisions by the asymmetric hair division, gives the root more root hairs than it would have had if the cells had had to complete the full sequence of formative and proliferative divisions in the cell lineage, assuming a maximum of 55 cell generations. A similar over-riding of formative divisions in the stele or cortex would not be advantageous as it would not lead to continuous cell files.

Cell cycle durations have been found to vary with cell types and position in roots (Clowes, 1975a; Barlow, 1976) and in the shoot apex of Polytrichum (Hallet, 1972). In Azolla the cell cycle time can vary not only between different cell types but can also vary for any given cell type, depending on the age of the root. Prolongation of the apical cell cycle with aging was observed for the ferns, Ceratopteris (Chiang and Gifford, 1971) and Pteris (Michaux, 1971; Michaux-Ferrière, 1975).

2.4.4 Control of Cell Division and Differentiation

Except in late stages of growth, zones of cell extension separate the sites of the successive rounds of transverse division. In the endodermis layer, for example, the initial cells elongate during
an extended lag phase (Table 2.1). Cell length then diminishes because the continuing elongation does not keep pace with 3 rounds of division. Next, further elongation and a 4th round of division proceed in balance (Fig. 2.54), prior to the final phase of elongation, which is accompanied by cell differentiation but not division. The extent of elongation in this final phase is not uniform along the root (Fig. 2.42), merophytes closer to the apex undergoing less elongation, as also observed in *Zea mays* by Erickson and Sax (1956).

In seeking evidence concerning the regulation of sites of transverse division (see Barlow, 1976), it seems significant that cell extension is a continuous process that transcends distinctions between cell layers, while cell divisions are discontinuous and cell-specific. The evidence provided by abnormal epidermal cells that proliferate precociously, presumably because of their greater initial length, suggests that elongation may also be causal for this category of division. Cell expansion is driven by turgor forces and shaped by cell-specific wall properties (Green, 1962). Transverse cell divisions and at least some formative divisions may be keyed to expansion (primarily to elongation), each cell type having its own successive thresholds for division, and its own permitted total number of rounds of division, after the completion of which terminal differentiation
begins while elongation continues.

The transverse, proliferative divisions in *Azolla pinnata* roots yield identical progeny, except possibly in the inner cortex, where two rounds of transverse division produce four progeny but the acroscopic member in the merophyte (in most roots) displays individuality by dividing again while the other three do not. Whether or not this extra division occurs, all members of the file become typical vacuolated cortex cells. Slight asymmetry in the placement of the walls in the first two rounds of transverse division was observed and this may be sufficient to trigger an extra, localised division.

Once the stele has been formed it changes very little in diameter; most of the increase in girth of the root is generated by vacuolation and lateral expansion in the two cortical layers (Figs. 2.13-2.18). It is important to examine wall development in these cells to determine what enables them to expand as well as to elongate.

The orderly progression through sequences of formative divisions indicates an ability of cells to sense the developmental stage reached by their older and younger neighbours, and to programme or be programmed accordingly. Cells cannot fulfil their role as initials until they have passed through the correct sequence of formative cell divisions.
In old roots neither the proliferative divisions nor vascular differentiation penetrate into the zone at the tip of the root in which a residue of cells that have not completed their lineages has been left. If the precursor cell for the inner stele has not completed division 8, it is incompetent to proceed along the pathway of xylem differentiation (see also Shinginger, 1975; Meins, 1975). At variance, there is the behaviour of division 10, which generates trichoblasts which can penetrate merophytes in which the two rounds of transverse division in the dermatogen (after formative division) have not been completed and can even progress into the formative zone, as discussed above. Recent evidence indicates that parenchyma cells can differentiate into tracheary elements without the intermediacy of cell division (Shinginger, 1975; Torrey, 1975; Turgeon, 1975), which was previously thought to be necessary (Torrey et al., 1971; Meins, 1975). In *Azolla*, it was not possible to determine, in the cleared roots, if the abnormal xylem elements which differentiated (Figs. 2.38-2.40), did so from cells that would normally have become inner sieve elements or phloem parenchyma cells. The susceptibility of the phloem parenchyma to undergo isolated, irregular divisions is apparent in two of the roots which were serial sectioned (Figs. 2.37, 2.43B,C), however it is not known if a similar division preceded the differentiation of the extra xylem elements.
Observation of transversely sectioned material would elucidate this point.

The distribution of cell types within the root is strongly correlated with the direction of apical cell cycling. Division 9 is radial, giving sister cells that at first look identical (Fig. 2.11). They differ in positional terms, however. If the helix of merophytes is followed from the apex, either to the left or to the right as the case may be, the cell that is reached first becomes a sieve element and its distal sister a small pericycle cell (Figs. 2.12, 2.14-2.17, 2.41). What these obligatory relationships with the handed-ness of the apical cell mean in terms of positional information or cyto-differentiation is a matter for speculation and an opportunity for experimentation.

The special morphogenetic roles of asymmetrical divisions have often been emphasised (Sinnott, 1960; Cutter and Hung, 1972). However, in Azolla the only obvious difference between the large and small progeny of the asymmetric division, 2a, is that the former re-divides (symmetrically) to add two more cells that appear very similar to the latter, all three being precursors for the dermatogen and the outer cortex. Conversely, Azolla roots provide an example of a symmetrical division (division 9) that generates sister cells with very different differentiative fates, i.e. one cell differentiates to become a sieve element, while the other cell
becomes a pericycle cell. In these cases symmetry and asymmetry as such are less significant than other factors, internal and positional, that control differentiation.

The *A. pinnata* root has thus been fully categorised in terms of the sites and sequences of divisions which generate every cell in the root and, at the same time, the study has given new information on the regulation of morphogenetic processes in plant cells. This knowledge will be used, in the remainder of this thesis, as a basis for studies of the microtubule arrays which occur in the cortex of the root cells.
TABLE 2.1. The site of completion of categories of formative division in roots of different stages of development (left hand side of Table), and the cell cycle durations for formative and certain transverse divisions (right hand side of Table). Cell cycles are measured in terms of the number of merophytes between completion of one division and completion of the next division, one merophyte being equivalent to one apical cell cycle. Odd numbered roots were associated with branches growing out to the right, viewing the frond from above.

<table>
<thead>
<tr>
<th>Type of Division</th>
<th>Merophyte number where each type of division was first seen</th>
<th>Type of Division</th>
<th>Number of merophytes for each successive cell cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(youngest merophyte = no. 1)</td>
<td></td>
<td>Position of root along frond (1-5) (oldest-youngest)</td>
</tr>
<tr>
<td></td>
<td>Position of root along frond</td>
<td></td>
<td>Position of root along frond (1-5) (oldest-youngest)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Formative</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>2a</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>2b</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>4a</td>
<td>?</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
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<td>16</td>
<td>16</td>
</tr>
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<td>10</td>
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<td>14</td>
</tr>
<tr>
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<td>17</td>
</tr>
<tr>
<td>12</td>
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<td>18</td>
<td>19</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Transverse</td>
<td>Dermatogen 1st</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Outer Cortex 1st</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Inner Cortex 1st</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Endodermis 1st</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>

Direction of helix: right right right left right
**Table 2.2** Merophytes in which the various categories and rounds of transverse division occurred in a 1.7mm long root of *A. pinnata*. Merophytes are numbered from the apical cell, the most recently formed = no. 1. The three sets of figures in each column represent the three 120° sectors of the root.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatogen</td>
<td>16</td>
<td>14</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Epidermis</td>
<td>31</td>
<td>29</td>
<td>33</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>Outer cortex</td>
<td>16</td>
<td>14</td>
<td>15</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Inner cortex</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Endodermis</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Large pericycle</td>
<td>16</td>
<td>?</td>
<td>18</td>
<td>28</td>
<td>?</td>
</tr>
<tr>
<td>Small pericycle</td>
<td>19</td>
<td>17</td>
<td>?</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Outer sieve element</td>
<td>16</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inner sieve element</td>
<td>-</td>
<td>17</td>
<td>15</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td>Parenchyma</td>
<td>19</td>
<td>17</td>
<td>-</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>Xylem</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Transverse division only in most apical cell of each file of four.
- Cell does not exist, or division does not occur.
FIG. 2.1 Measurement of the root lengths of *Azolla pinnata* plants growing in 1/50th dilutions of Hoagland's solution showed that this medium did not sustain good growth of Australian material. Transfer of the plants to fresh medium at day 8 and day 15, stimulated the elongation of some roots.
FIGS. 2.2-2.4 *Azolla pinnata* R. Br.

**Fig. 2.2** The green pigmentation of the actively-growing plants in this colony contrasts with the red colouration of the plants in the dam near Bega, N.S.W. (Fig. 1.1). Approximately 4 times natural size.

**Figs. 2.3-2.4** The growing apex of a plant viewed from above (Fig. 2.3) and below (Fig. 2.4). Leaves, branches and adventitious roots arise from branch points along the frond, alternating from left to right. The young roots are enclosed within a sheath and a 2-layered root cap. Root elongation is rapid after the stages shown here. Roots pointing to the right in the view in Fig. 2.4 possess a counter-clockwise segmentation, those to the left, a clockwise segmentation. X20.
**Fig. 2.5** Sporocarp production. Plant viewed from below, possesses two male, microsporocarps (arrows). The period of active growth of the colonies of *A. pinnata* plants was limited and, at the end of this period, plants produced micro- and macro-sporocarps, after which the whole colony would die. Approx. X8.

**Fig. 2.6** The effect of growth medium on plant form. The basal portion of this frond (bracket) was produced when the plant was growing in pond water. Transfer of the plant to the nitrogen-free culture medium promoted growth of the lateral branches. Approx. X5.

**Figs. 2.7-2.8** Development of roots in *A. pinnata*. Initially the root proper is hidden within a sheath and a 2-layered cap (root h, Fig. 2.8). Root elongation breaks off part of the sheath (at arrows) and later on releases root hairs from confinement within the cap (roots b and d, both figures). The cap is sloughed off when root elongation has ceased (roots a and c, Fig. 2.7; root a, Fig. 2.8). The root hairs near the root apex, are shown after premature removal of the cap (roots c, e, g and i, Fig. 2.8). Approx. X4.
FIGS. 2.9-2.18 Cell division and development in *A. pinnata* roots.

FIGS. 2.9-2.11 Transverse sections at different levels in the zone of formative divisions in a root 0.7 mm in length.

**FIG. 2.9** Only the older of the 3 merophytes at this level of the root has completed the final tangential, longitudinal divisions which yield 2 inner cortex (ic) and 2 endodermis (e) cells.

**FIG. 2.10** Further from the apex, the older of the 3 merophytes has completed the third tangential, longitudinal division in both 60° sectors. Inter-merophyte walls, at 120° to one another (arrow heads) are characterised by the intercellular space between the inner and outer cortex (oc) layers.

**FIG. 2.11** The radial, longitudinal division (arrowed) has been completed in the cell internal to the endodermis, in one 60° sector. The division is symmetrical and it cannot be immediately distinguished which of the 2 daughter cells will become an outer sieve element cell and which a small pericycle. In a root of this length, the asymmetrical root hair division has not progressed along the file of dermatogen cells (d) into this region of the formative zone. Figs. 2.9-2.11, X570.

**FIGS. 2.12, 2.14-2.17** Transverse sections in regions of cell proliferation and differentiation. The roots in Figs. 2.12, 2.14 and 2.16 are clockwise, those in Figs. 2.15 and 2.17 are counter-clockwise. Wall thickening (white and black arrows) is evident in the corners of the differentiating outer sieve element (os) and xylem (x) cells. The asymmetric root hair division has been completed in these more mature regions of the roots, yielding root hair (r) and epidermal (ep) cells. The wall ingrowths which form as xylem differentiation nears completion are shown at an end wall (Fig. 2.15) and at the central wall (Fig. 2.17) [arrow heads] Differentiation of the inner sieve element (is) has occurred near the base of a 5 mm long root (Fig. 2.17). In older regions of the roots the endodermal cells become more rounded and the xylem elements, assume a more trapezoid shape (Figs. 2.16, 2.17). p - pericycle; pp - phloem parenchyma. Figs. 2.12, 2.14, X570; Figs. 2.15-2.17, X900.

**FIGS. 2.13, 2.18** Longitudinal sections of a 0.8 mm long root (Fig. 2.13) and a 1.4 mm long root (Fig. 2.18). Some of the transverse inter-merophyte walls near the root apices are marked by the arrow heads. Sites of the first representatives of longitudinal walls can be seen, as can sites of the rounds of transverse divisions. The first root hair division (r) occurs in a merophyte containing 4 dermatogen cells in the root in Fig. 2.13, but has overtaken the second transverse division in the dermatogen in the older root (Fig. 2.18). The sites of transverse divisions are also closer to the apex in the older root, e.g. the first transverse division of the epidermis (ep). Fig. 2.13, X270; Fig. 2.18, X430.
Figs. 2.19-2.29 The apical cell and the first formative divisions.

Figs. 2.19-2.22 Transverse views of a young root, using Nomarski interference-contrast optics, focusing at increasing distances from the apical cell (Fig. 2.19). Division of the apical cell yields three merophytes occupying 120° sectors of the root (Fig. 2.20). The first intra-merophyte division is curved and tangential, longitudinal and divides the merophyte into an inner and outer layer (Fig. 2.21). The first radial, longitudinal division in the outer layer is asymmetrical, giving a 40° sector and an 80° sector (Fig. 2.21). The latter then divides symmetrically, giving 3, 40° sectors in the outer layer of each merophyte (Fig. 2.22). The inner cell in each merophyte also divides in a radial, longitudinal plane, to give 2, 60° sectors (Fig. 2.22). All X750.

Figs. 2.23, 2.24 Transverse sections through apical cells in a very young root (Fig. 2.23) and a 0.9mm root (Fig. 2.24). The apical cell has become highly vacuolated in the older root. Both X700.

Figs. 2.25, 2.26, 2.29 Longitudinal sections of very young roots. The root in Fig. 2.25 contains only 7-9 merophytes. The next root along the frond is shown in the electron micrograph in Fig. 2.26 and in the light micrograph in Fig. 2.29, and contains approximately 15 merophytes. Fig. 2.25, X400; Fig. 2.26, X2,000; Fig. 2.29, X700.

Figs. 2.27, 2.28 Transverse sections of a 0.9mm root at levels comparable to those in Figs. 2.21 and 2.22 respectively. One merophyte in the section in Fig. 2.28 has completed the first tangential, longitudinal division in the innermost sextant cells. Both X700.
Figs. 2.30-2.32  The apical cell and continued merophyte production; longitudinal sections.

The root containing approximately 21 merophytes (Fig. 2.30) occurred adjacent to the root in Figs. 2.26 and 2.29. The 30-merophyte root (Fig. 2.31) also branched off the same frond, and was adjacent to that in Fig. 2.31, i.e. the roots in Figs. 2.25, 2.29, 2.30 and 2.31, were sequential roots along a single branch.

The apical cell continues to divide and produce the 3 tiers of merophytes until the root contains approximately 55 merophytes. The first divisions within the merophytes are in the longitudinal plane and generate cell initials for every category of cell in the root, except the root hair and epidermal cells (see Figs. 2.12-2.18). The first representatives of the longitudinal walls and the sites of the first rounds of transverse divisions are much further from the apical cell in these young roots than in the more mature roots in Figs. 2.13 and 2.18.

The apical cell in the root in Fig. 2.31 was in the process of forming a transverse cell plate (Fig. 2.32), the completion of which would have formed the 11th merophyte along that 120° sector. Fig. 2.30, X2,600; Fig. 2.31, X500; Fig. 2.32, X6,300.
FIGS. 2.33-2.36 Determination of the sequence of formative divisions by serial transverse sections.

The four transverse sections from a sequence of serial sections through the formative zone of a 0.7mm root illustrate the method used to determine the divisions which had occurred within each merophyte. Merophytes have been numbered sequentially, the youngest (number 1) being that most recently produced by division of the apical cell (Ap). Inter-merophyte walls have been traced by a solid line; intra-merophyte walls are delineated by a broken line. The apical cell shows counter-clockwise segmentation.

Progressing away from the apical cell along the sequence of sections, older merophytes "appear" at outer edge of the root, then "move" towards, and "disappear", at the centre of the root (e.g. merophyte 1). These, and the subsequent, formative divisions are represented diagrammatically by Fig. 2.46. All X3,200.

FIGS. 2.37-2.40 Abnormalities of division and differentiation.

FIG. 2.37 The phloem parenchyma cell has completed an abnormal, oblique division (arrows). Serial sectioning showed that the acroscopic and basiscopic merophytes did not possess this abnormality. The root had been pulsed for 24 hours with 5-Aminouracil, 14 hours before fixation. X1,900.

FIG. 2.38-2.40 Nomarski interference-contrast optics views of cleared roots from a colony of plants growing in nitrogen-free culture medium. The normal files of xylem elements are not continuous and isolated cells in other files in the inner hexagon of the root have also differentiated to become xylem elements. Figs. 2.38, 2.40: X400; Fig. 2.39: X660.
Fig. 2.41 An *Azolla pinnata* frond, viewed from under water, showing the ventral surfaces of the two ranks of shoot merophytes. Hypothetical successive stages of root apical cell formation are diagrammed (Strasburger [1873] and Campbell [1905] showed that root apical cells arise in these ventral regions). In stages a-c and a'-c' three successive divisions lay down walls 1-3 which form the three proximal faces of the apical cells. The sequence 1-2-3 is extrapolated without change in direction when the apical cell starts to cycle (stages d and d', walls 4-5-6). Divisions in a and a', b and b', c and c' are geometrically equivalent, but occur in merophytes that face in opposite directions, hence clockwise and counter-clockwise apical cells are produced. In stages d and d' the arrows start at the youngest merophyte in the new root and follow the helix towards the oldest. In stages e and e' the cell dispositions that impose 2-fold rotational symmetry are shown superimposed on the outline (dotted) of the apical cells. The xylem axis (x-x') lies in the anterior-posterior axis of the shoot. Positions of outer sieve elements (s) and their sister cells, and of xylem elements are shown in relation to the radial inter-merophyte walls in the root (solid lines) and the sextant walls (dashed lines) and in relation to the direction of the helix of merophytes (short arrows).

Fig. 2.42 Merophyte elongation. The extent of elongation of the merophytes is indicated by the distance between the root hairs along the roots. This distance increases further from the root apex. Merophytes are approximately 3-4 µm in length (in the longitudinal direction) when produced by division of the apical cell. The maximum distance between root hairs is approximately 1.1 mm, in the longest root, and as each merophyte in this region of the root would contain 4 transverse rows of root hairs, a 1,000-1,500-fold increase in merophyte length has occurred. Root lengths: ▲ 46 mm; ● 35 mm; ◇ 21 mm; □ 15 mm; ○ 10 mm.
Fig. 2.43  Diagrams of cell divisions in successive merophytes (1, 2, 3, ... ) proximal to the apical cell (itself not shown).

Row A: A theoretical situation in which every successive cell cycle, irrespective of its type, takes the same length of time as the apical cell cycle. It would take only 8 merophytes to complete the lineages.

Row B, C, D: Reconstruction of events in actual roots, obtained by serial transverse sectioning (illustrated in Figs. 2.33-2.36). Roots in rows B and C are clockwise, that in row D is counter-clockwise. The root in row B is the longest of the three and the formative divisions have occurred closer to the root apex. The positions of pre-prophase bands observed in the roots are indicated in rows C and D by the dots. Note the variation of position of division 2a (see Fig. 2.46): towards the next younger merophyte in rows B and C and towards the next older merophyte in row D.
Fig. 2.44 (left). Formative division types are shown ranked according to the order in which they occurred in 10 roots, the order being based on the number of the merophyte in which the division was first seen. If two divisions (e.g. numbers 2a and 3) occurred at the same time (within the limits of resolution of the transverse serial sectioning method that was used), the symbols were placed on the line between the successive compartments of the matrix.

Fig. 2.45 (right). Longitudinal sections were used to determine the relative order in which selected categories of transverse, proliferative divisions were initiated. Only the oldest roots in the sample that was studied had reached the stage of having the final rounds of division in the endodermis and outer cortex.

Fig. 2.46 Diagram summarising the formative division stages and the final outcome of the proliferative divisions. Segmentation of merophytes by the cell lineage divisions (numbered as in Fig. 2.47) is shown at left and along the top. The columns represent the final stage of the cell files within merophytes after cell proliferation. The small numbers indicate the number of rounds of division in each case.
FIG. 2.47 Cell lineage diagram for the formative zone of *Azolla pinnata*.
The code number for each division is circled; below the circles the letters t,r,l and o refer to tangential, radial, longitudinal and oblique planes of division; S and A refer to symmetrical and asymmetrical divisions respectively.
Figs. 2.48-2.52 Changes during early stages of root development in *A. pinnata*.

**Fig. 2.48** The width of young roots, as measured by the outer diameter of the root cap.

**Fig. 2.49** The total number of merophytes per root reaches a plateau after about 55 apical cell division cycles, accomplished by the time the root is less than 1mm in length.

**Fig. 2.50** Daily measurement of the lengths of roots at successive branch points along a single frond. The rate of elongation increases markedly in roots greater than about 0.7mm in length.

**Fig. 2.51** The length increments in Fig. 2.50, and from other replicate fronds, were combined and show that the rate of root elongation is dependent on the length of the root.

**Fig. 2.52** Apical cell cycle durations were calculated by counting the number of new merophytes between adjacent roots along the fronds and by estimating the time interval between the stages of development of the adjacent roots, using Fig. 2.51.
Fig. 2.53 Changes during early stages of development in *A. pinnata* roots.

The sites at which successive transverse divisions in cell files (marked 1st, 2nd....), and root hair formation, occur. The scattered points in the case of the 3rd round in the inner cortex (D) arise from the erratic occurrence of this division, found only in the acroscopic member of each file (see text).
FIG. 2.54 Representative data illustrating the relative constancy of mero-phyte lengths in which successive rounds of transverse division in the outer cortex and the endodermis are initiated. Symbols as in Fig. 2.53.
Abnormalities of cell division detected by serial transverse sections through the formative zone of the root.

Diagrams of cell division in successive merophytes proximal to the apical cell in 3 roots which were found to possess abnormal divisions (compare with Fig. 2.43).

Row A. Although division 3 has not occurred in merophyte 10, two tangential, longitudinal divisions (which would normally be divisions 5 and 7) have been completed. Positions at which pre-prophase bands were observed are indicated by the dots. Root, counter-clockwise.

Row B. In merophyte 11 the normal tangential, longitudinal division in the central dermatogen - outer cortex precursor cell has not occurred but a radial, longitudinal division has been completed precociously. In merophyte 12, an extra division has occurred in the phloem parenchyma cell. Root, clockwise.

Row C. An extra division has occurred in the phloem parenchyma cell in merophyte 15. This root was fixed 14 hours after a 24 hour 5-Aminouracil pulse. Root, counter-clockwise.
CHAPTER 3
THE STRUCTURE OF CORTICAL MICROTUBULE ARRAYS IN
PLANT CELLS

After completion of the work described in this Chapter, my colleagues kindly estimated that the 32,000 microtubule profiles that had been counted in the analysis represented a total microtubule length of 2.2mm, depicted below, the width being magnified 20,000 times!

"It has long been an axiom of mine that little things are infinitely the most important."

(Sherlock Holmes) Sir A. Conan Doyle

3.1 INTRODUCTION

The discovery of cortical microtubules in plant cells followed the introduction of glutaraldehyde as a fixative for fine-structural studies. Ledbetter and Porter (1963) described them as being "arranged circumferentially [against the lateral walls of root tip cells]....like hundreds of hoops around the cell", parallel to microfibrils of cellulose in the cell wall. While admitting that individual microtubules could only be followed for short distances, Newcomb (1969) stated that "In some cases they run circumferentially around the cell in the cytoplasm near the plasmalemma, probably forming complete rings or hoops". Green et al. (1970) also considered that cortical "microtubules are arranged in a very loose spiral or are present as rings". Srivastava and Singh's description (1972a) of microtubules overlying developing xylem thickenings is that they too "run like hoops around the cell", and
Hepler and Palevitz (1974) refer to those in a differentiating sieve element as being "arranged hooplike around the cell". Pickett-Heaps also (1974) envisaged that "cytoplasmic microtubules normally encircle the cell transversely to the long axis in both higher plants and many algae". Even more recently Schnepf et al. (1976) implied that the cortical microtubules are long in suggesting "unusually fast elongation of the microtubules [along a newly-formed wall] so that they soon appear on the opposite wall....".

There is, however, growing evidence that microtubular arrays can contain overlapping rather than full length microtubules (Fuge, 1974; Heath, 1974b; Heath and Heath, 1976; Jensen and Bajer, 1973; Lambert and Bajer, 1972; McIntosh, Cande and Snyder, 1975; McIntosh, Cande, Snyder and Vanderslice, 1975; Singh and Srivastava, 1973; Warren, 1974). In the case of the plant cell cortex this might be of especial importance. It has become evident that the congruent alignment of cortical microtubules and wall microfibrils is widespread during primary and secondary wall deposition (see Chapter 1) and any attempt to specify functional aspects of this co-orientation is crucially dependent upon knowledge of the actual three-dimensional arrangement of the microtubules. At least one hypothesis views the microtubules as providing rigid guide tracks for the movement of cellulose synthetase complexes (Heath, 1974a). Yet, there is in fact no compelling evidence that cortical microtubules are long hoops or helices. Warnings given by the original discoverers that they could equally be in the form of overlapping arcs (Ledbetter, 1967; Ledbetter and
Porter, 1970) have gone largely unheeded.

Trends in the distribution and orientation of microtubules can be ascertained from individual sections which contain transverse or longitudinal profiles, but neither view allows precise determination of microtubule lengths or of the overall form of microtubule arrays. In longitudinal section, microtubule terminations (if present) cannot be distinguished unequivocally from microtubules that are passing out of the surfaces of the sections. Transverse profiles can, however, be used to track microtubules throughout sequences of serial sections and this has been the approach employed here. The main aim was to provide basic information on the geometry of cortical microtubule arrays in plant cells, this being fundamental to an understanding of how they are formed and maintained, and how they might function.

3.2 MATERI AL S AND METHODS

3.2.1 Plant Tissue and Routine Fixation

Root tips of *Azolla pinnata*, *Impatiens balsamina* and *Zea mays* were fixed and then embedded in Spurr's resin as outlined in section 2.2.2. Variations from this standard procedure are described in Table 3.2 and appropriate parts of the Results section. Most of the work employed root tips of *Azolla pinnata*. These have the advantages of being very small, fixing well, and being suitable for treatment with aqueous solutions. The cellular construction of the root is unusually precise,
every stage of development of every cell type can be categorised and comprehensive background information on the development of these roots has been presented in Chapter 2. Not all the cells that were serially sectioned were identified; they included developing sieve and xylem elements, and endodermis and cortex cells.

3.2.2 Experimental Treatments

For low temperature treatments, intact *Azolla pinnata* plants were transferred to culture medium in beakers surrounded by ice, in a cold room, for either 15 minutes or 4 hours. The temperature of the water in which the roots were immersed varied within the range 0-2°C in different experiments (Table 3.7). Roots were fixed for 1 hour at this temperature and then for a further 1 hour at room temperature. In a recovery experiment, roots were transferred to room temperature culture medium for 15 minutes before fixation at room temperature. The rate of cooling and warming in the actual root tissue was not measured, but since the diameter of the root at the level that was analysed is only approximately 150µm, it is assumed to be rapid. Treatments with colchicine in the dark at $5 \times 10^{-3}$M lasted 2, 3, 4 or 5 hours, as well as 2 hours treatment followed by a 1 hour recovery. Deuterium oxide (D$_2$O) was used at 99.8% purity for
for 5 or 18 hours before fixation (in glutaraldehyde dissolved in D_2O). Pressures of 6,000 psi (for 30 minutes), 14,000 psi or 16,000 psi (for 15 minutes) were applied to intact Azolla pinnata plants using a French pressure cell. The pressure was released smoothly but quickly and roots immersed in fixative within 30 seconds of the onset of decompression.

3.2.3 Serial Sectioning

Serial sections from small block faces of longitudinally oriented roots were collected on parlodion-coated grids with 2mm slots. Ribbons were rejected if they did not contain clear transverse profiles of cortical microtubules in every section of a particular cell, as were ribbons with missing or damaged sections. Suitable preparations were photographed at 30,000X and printed to a final magnification of 75,000X.

3.2.4 Tracing and Mapping

Microtubule profiles were traced from the photographs of each serial section onto cellulose acetate sheets. Individual microtubules were followed by placing the tracing of one section over the photograph of the adjacent section in the sequence. When the entire sequence had been analysed in this way and the positions of terminations and the paths of the microtubules determined,
the position of each microtubule profile on the tracings was plotted on graph paper so as to build up a map of the array in the plane at right angles to the plane of sectioning (e.g. Figs. 3.13, 3.15, 3.20). Terminations were arbitrarily taken to occur halfway through the section in which they had been detected. Where possible, a microtubule which continued right through the sequence was used as a reference and each successive section aligned with respect to it. Spatial separation of microtubules in the direction from the wall to the centre of the cell cannot be depicted on these maps, and some microtubules which appear to lie very close together may, in fact, be some distance apart. In preparing the maps of some very dense arrays, it was necessary for the sake of clarity to displace the positions of a few microtubules just enough to show their existence.

3.2.5 Estimation of Section Thickness

The construction of the microtubule maps described above and the calculation of microtubule lengths is dependent on knowledge of the section thickness. The methods that have been developed for the estimation of the thickness of ultra-thin sections (Williams and Meek, 1966; Sjostrand, 1967; Small, 1968; Hayat, 1970; Gillis and Wibo, 1971; Reid, 1974; Casley-Smith and Crocker, 1975) relate to individual sections, and the results obtained
are influenced by a number of factors, including the degree to which the sections are spread by either heat or solvent vapour treatment after being floated off the knife edge, and the extent of thinning due to exposure to the electron beam. These points, together with the inevitable variation in section thickness along ribbons of adjacent sections, and uncertainty as to whether material is lost as the knife cuts through the block, limit the value of the published methods for application in the construction of three-dimensional representations from superimposed micrographs. However, a simple means of determining the average section increment along ribbons of ultra-thin sections was developed, calibrating the ultramicrotome used in the present work by estimating the depth to which ribbons of ultra-thin sections of various interference colours cut into the block.

Using a glass knife and the angular graduations marked on either the knife holder or block holder assemblies of the microtome, the block was trimmed so that the face surmounted pyramidal slopes of known angle, θ (Fig. 3.1a). A Reichert OMU3 ultramicrotome was used to trim the blocks for determinations with values of θ greater than 60°. The LKB Pyramitome offers a greater choice of angle and as ultra-thin sectioning was found to be more difficult at angles greater than 55° the Pyramitome was used to obtain values of θ less than
The smaller the angle that is used, the larger the number of sections that need to be cut to maintain the same order of accuracy.

The linear dimensions of the sides of the block face were measured using a calibrated optical microscope set up so as to give the highest possible magnification (and hence greatest accuracy of measurement). The block was simply held between the arms of a pair of forceps which were rested on the mechanical stage assembly, and the sides were measured by means of an eyepiece micrometer, using a X40 objective.

The block was carefully realigned in the ultramicrotome so that cutting commenced in a plane exactly parallel to the block face, avoiding taking off any thick sections, and a long ribbon totalling, say, 200 sections was cut. The block face was measured (Fig. 3.1b) and the average increase in length of its sides determined. The average section increment was calculated as indicated below the figure. For example, in one case the four sides of the block face initially measured 0.157mm, 0.114mm, 0.157mm, 0.114mm. After cutting 170 sections the sides were then 0.184mm, 0.137mm, 0.184mm and 0.141mm respectively. This gives an average increase of 0.026mm and thus the average section section increment ($\theta = 45^{\circ}$) = 76nm.

This method was applied to obtain the average section increment in ribbons of sections.
of different thicknesses as indicated by the interference colours given by the sections when floating in the knife bath. The results obtained are listed in Table 3.1 as average section increments, since the method does not determine individual section thicknesses, nor whether material is lost between adjacent sections. To investigate these matters and to provide a cross check on the results, the value of the average section increment was compared with the average section thickness given by an alternative method.

The sections cut in two of the series listed in Table 3.1 were collected on slot grids and the thickness of the individual sections was determined using an interferometric method (modified from Gillis and Wibo, 1971). Each section was photographed using a Leitz Orthomat interference microscope (Mach-Zehnder system) so that the interference fringes passing through the sections and their support film could be seen in the field of view. The 35mm negatives obtained in this way were further enlarged onto sheet film and the images of the fringes for each section were then scanned by means of a densitometer-chart recorder attachment to a Gilford spectrophotometer. The fringe shift, at an amplification of X33 relative to the original 35mm negatives, was measured and the section thickness was calculated from the relationship:
section thickness = \frac{\text{shift (nm)}}{\text{refractive index (resin) - refractive index (air)}}

The refractive index of the Spurr's resin was determined as in Gillis and Wibo (1971), and the value of 1.508 obtained.

Analysis of 50% of the 165 sections cut in one series gave a value for the average section thickness of 82.6nm. This is to be compared to the value of 82.3nm obtained using the block face method. The interferometric method also showed that there was very great variation in section thickness. Measurements of 83 sections along one ribbon, and 40 in another, gave coefficients of variation of 34.1% and 32.7% respectively. No doubt this coefficient would vary from one ultramicrotome to another, as well as with other operating conditions. It was concluded that the simple procedure based on measuring the block face yields a reliable estimate of the average section thickness, and that it is in fact likely to be more reliable for this purpose than procedures based on determinations of thickness in small samples of individual sections from a ribbon. An average section increment of 70nm was estimated for the ribbons of serial sections used in the present work.
3.2.6 Derivation of the Average Microtubule Length

Consider the microtubules to be arcs lying adjacent to the walls of a cylindrical cell, perimeter $A$, as viewed in transverse section in Fig. 3.2. The average length of the microtubule arcs, $L$, is assumed to be less than $A$. The cell is sectioned longitudinally and the total thickness of the combined serial sections is the sample arc, $a$. The lengths are assumed to be independent of position and of each other, the midpoints of the microtubules forming a Poisson process of intensity, $\lambda$. In this situation, the expected number of terminations ($T$) occurring in the sample arc is given by $T = 2\lambda a$. The expected number of microtubules ($N$) passing through a fixed point is $N = \lambda L$, and hence the average microtubule length can be calculated from the relationship $L = 2Na/T$. An intuitive derivation is given in the Results (Section 3.3.5) and the validity of the formula is easily checked using imaginary arrays drawn on graph paper. The use of sections of finite thickness introduces an error which can be avoided if, when counting the number of profiles of microtubules per section in order to derive $N$ (the average number per section), one unit is added for each tubule that is not terminating, and one half unit for each tubule that terminates in that given section. Thus it is assumed that the terminations always lie at the mid plane of the sections.
3.2.7 Analysis of the Spatial Distribution of Terminations

Objective methods for detecting possible non-randomness in the distribution of terminations were applied. A multiple $\chi^2$ test was used to compare the observed distribution of terminations amongst the sections in each sequence with that predicted by a Poisson distribution calculated using the average number of terminations per section as its mean. The significance of calculated values of $\chi^2$ was determined from the table given in Fisher (1948). Arrays described in the Results (Section 3.3) as "random" all had probabilities of correspondence with Poisson distributions $> P = 0.05$ (mostly $>>$) and of these described as "non-random" the probabilities of correspondence were $< P = 0.01-0.02$ (mostly $<< P = 0.01$). This analysis however indicates deviation from randomness only if the non-randomly distributed terminations lie in a pattern that is detectable by the sampling procedure, i.e. by the cutting of sections in a particular plane. Evidence for non-randomness due to clumping of terminations over several successive sections, or to lines of terminations not lying in the plane of the section, may be obtained by applying a pattern analysis which measures the variability or spread of a distribution of points (Greig-Smith, 1952; Kershaw, 1973). In this procedure, a microtubule map is subdivided by a grid to give convenient
grid units or "blocks" and the number of terminations in each block is counted. If the distribution of terminations is random, the variance is equal to the mean, whereas larger values of variance indicate clustering and lower values regular dispersion. The variance of the population is calculated at different scales, i.e. at different unit block sizes, and the ratio of variance to mean is plotted against block size. Confidence bands (Thompson, 1958) are applied to determine size scales at which the distribution deviates significantly ($P = 0.05$) from randomness. Similar analyses have been performed to examine the distribution of ribosomes in plastids (Brown and Gunning, 1966) and of nuclear envelope pores (Lott et al., 1972).

3.3 RESULTS

3.3.1 Microtubule Terminations in Longitudinal and Transverse Views

If an ultra-thin section is 75nm (i.e. 3 microtubule diameters) thick, any microtubule whose long axis deviates from the plane of section by as little as $4^\circ$ will only have, at most, $1\mu$m of its length included within the section. The zone over which the microtubule leaves (or enters) the surface of the section will show a gradual loss of image of the tubule; Figs. 3.3 and 3.4 which show two adjacent serial sections through a pre-prophase band of microtubules, contain many examples. In theory,
it should be possible to follow microtubules in longitudinal view from one section to another until terminations are found. In practice, it is very difficult to do so, especially in dense arrays of microtubules. However, where the microtubules lie at right angles to the plane of section, the relative positions of the circular transverse sectional profiles allow the presence or absence of a particular microtubule to be determined with confidence (Figs. 3.3, 3.4).

Eleven adjacent sections from one sequence of serial sections are shown in Fig. 3.10. Examples of microtubule terminations can be seen by following the numbered microtubules. Eighteen of the 42 microtubules that are present in this part of the sequence end within the sections shown, the terminations occurring throughout the array. The sequence includes one short complete microtubule which begins in section c and ends in section j. Occasionally a microtubule contains a zone spanning several sections in which the staining is much fainter than in the neighbouring regions, other microtubules and cell organelles near these fainter profiles nevertheless being normally stained; an example is arrowed in section d of Fig. 3.10.

No electron-dense material was consistently seen at termination sites, but 1.7% of the terminations were found to possess a C-shaped profile
in transverse section (Table 3.6), as exemplified by microtubule number 2 in section a of Fig. 3.10. This conformation usually occurs in the last one or two sections but has been seen for up to 14 sections (1µm) from the end of the microtubule. On two occasions microtubules were found to possess C-shaped profiles which were isolated from the terminations by complete, circular profiles. Sometimes the arms of the "C" are much reduced, leaving only a small arc of the original microtubule profile.

The 12 C-shaped terminations (1.7% of a total of 706) showed non-random distribution patterns within the arrays. They were present in just three of the sequences of serial sections (all from different cells), one having 2 and the others 4 and 6 respectively. C-shapes were never found at both ends of an individual microtubule, and if one mentally symbolises a C-shape as an arrowhead at one end of a microtubule, then in the array that had two C-shapes, both "arrows" pointed in the same direction. In the array with 4 C-shapes, all 4 pointed in the same direction and the same applied to the array with 6 C-shapes. Within a given array the microtubules with C-shaped terminations evidently share a common directionality, at least within the portions of the arrays encompassed in the sequence of serial sections.
3.3.2 Bridges

Many examples of cross-bridges between adjacent microtubules and also between microtubules and the plasma membrane are illustrated in Figs. 3.3-3.10. The bridging between the longitudinally-sectioned microtubules in Fig. 3.4 exhibits a herringbone pattern with a periodicity approximating to the diameter of the microtubules themselves. Bridges attached to transversely-sectioned microtubules are not readily distinguished from other material that may be lying close to the microtubules. However, relatively clear images of putative bridges were seen in all three categories of cortical array: in pre-prophase bands (Figs. 3.3-3.10), in interphase arrays (Fig. 3.26), and in arrays of microtubules overlying developing xylem thickenings (Fig. 3.12). They are present on short as well as long microtubules. In a number of cases a cross-bridge either to the plasma membrane, or to an adjacent microtubule, occurred on the terminating profile of a microtubule. On occasions, more than one bridge connected a single microtubule profile to the plasma membrane or to adjacent microtubules (Figs. 3.6-3.9). Cross-bridges between microtubules in pre-prophase bands and vesicles were occasionally seen (Fig. 3.5).

Treatments which are known to stabilize microtubules or which can cause their depolymerization were used in parts of this study (see later):
following all such treatments the appearance and distribution of the microtubule cross-bridges was similar to that described above in control tissues.

3.3.3 Microtubule Maps

It is evident from the microtubule maps that in all three types of cortical microtubule array described below, some microtubules continue right through the sequence, others have one end within the sequence, and a few have both ends in the sequence, that is, the complete microtubules lie within the thickness of tissue sectioned. Some neighbouring microtubules maintain the same relative spacing over long distances, but many show undulations; some (usually lying deeper in the cytoplasm) pass at an angle to the remainder; and some leave one bundle and slant across to join another bundle. Many of the sequences of sections contained occasional microtubules lying at large angles to the majority, e.g. Fig. 3.10. These have not been included in the maps or calculations.

Differentiating Xylem Elements:

An early correlate of the initiation of wall thickenings in developing xylem is the appearance of groups of microtubules along the longitudinal walls (Pickett-Heaps, 1966, 1967b) and this was also seen in Azolla pinnata roots (Fig. 3.11).
In studying the development of xylem elements in *Azolla pinnata* roots it was found that thickening formation may be apparent on one side of the cell only (Fig. 3.11) [see section 4.3.3]. Similarly, it was often observed that microtubules were grouped on one side of the cell while they were distributed more evenly along the opposite wall. In some cases reconstruction of microtubule arrays in young xylem elements followed the convergence from a dispersed array to a pattern of groups, visible wall thickening commencing where the groups are clearly established (Figs. 3.11-3.13). In more fully differentiated xylem elements, possessing well-developed thickenings on both sides of the cell, the microtubules remain grouped over the thickenings throughout the sequences.

**Pre-prophase Bands:**

The occurrence of pre-prophase bands of microtubules throughout *Azolla* root tips is described in detail in Chapter 5. The number of microtubule profiles in a band varies widely, from 30 to >100 (Figs. 3.16, 3.17). Where the number is small, most lie close to the plasma membrane (Fig. 3.5); where it is large, the width of the band remains about 2µm and the microtubules become stacked (Figs. 3.6-3.9). The number can change (Fig. 3.15), sometimes markedly (Fig. 3.18), within a short sequence of sections. In the five pre-prophase bands that were serially sectioned, between 48% and 74% of the
microtubule terminations occurred near the plasma membrane. In general, this percentage approximates to the percentage of microtubules that lie adjacent to the plasma membrane. Further analysis of the positions of terminations in pre-prophase arrays shows that for 90% of the microtubules that were completely included in the sequences of sections, both terminations of any given microtubule lay at the same distance from the plasma membrane.

Interphase Arrays:

As in differentiating xylem elements, and in pre-prophase bands, the numbers of microtubules on opposite sides of an interphase cell are not always equal. While in most sequences of serial sections the numbers of microtubule profiles per section remained relatively constant (e.g. Figs. 3.22, 3.23A, 3.25A), a few cases were found where they slowly changed (e.g. Figs. 3.20, 3.25B). The array shown in Fig. 3.23B includes a very abrupt change. In this case eleven of the thirteen microtubules in section 24 terminated and only two continued into section 22. As the sequence continued, the number of microtubules slowly increased again. Fig. 3.23A contains a less obvious line of terminations at the same part of the sequence, the two arrays in Fig. 3.23 lying back-to-back less than 1µm apart in two adjacent interphase cells.
3.3.4 Analysis of the Distribution of Microtubule Terminations

The observed distribution of microtubule terminations throughout the sequences of sections was compared to theoretical distributions calculated for each sequence from Poisson series in which the mean is the number of terminations per section. In the majority of cases obtained from untreated root tips the observed dispersion of terminations did not differ significantly from the Poisson distributions, but in three interphase arrays and one pre-prophase band deviation from randomness was due to the presence of either: (i) clusters of terminations where most of the members of a parallel group ended within a few sections (as in two interphase arrays [e.g. Fig. 3.20], or (ii) linear arrangements of terminations (as seen in the interphase array in Fig. 3.23B and in the pre-prophase band). Other linear arrangements of terminations were seen, but since the line did not coincide exactly with the plane of sectioning, the overall agreement with the Poisson distribution was retained. Similarly, some microtubule maps gave visual impressions of clustering but because of their dispersion through the sections, the overall distribution of terminations did not deviate significantly from the Poisson series.

A pattern analysis which can show if a set of points is randomly distributed, clustered or
regularly dispersed was also used. When applied to the microtubule arrays, significant clustering was found to occur in all of the sequences which had, and three sequences which had not, deviated from the Poisson distribution. Thus, in seven of the sixteen arrays analysed in this part of the work, the distribution of microtubule terminations showed some form of non-randomness.

3.3.5 The Average Length of Microtubules

The sequences of serial sections and the maps drawn from them show that the observed microtubule lengths range widely from microtubules which are recognizable in only one section up to the longest so far detected, which was still continuing after 84 sections (5.9µm). Despite the variation in length, semi-quantitation is possible. There are two terminations for each microtubule in an array when the total thickness of the combined serial sections (a) just matches the average microtubule length (L). Sequences of sections that are shorter or longer than this will contain correspondingly fewer or more terminations:

\[
\text{Number of terminations in sequence (T)} = \frac{a}{2 \times \text{average number of microtubules per section (N)} \times L}
\]

A more rigorous derivation of this formula is set out in the Materials and Methods (Section 3.2.6). Strictly it should only be used where the distribution of
terminations conforms to a Poisson distribution. The data have, however, been pooled because the non-random arrays were not obviously different from the random in terms of calculated average length. In calculating microtubule lengths it was assumed that all microtubules lay at right angles to the plane of section and that the cell cortex was flat rather than curved: both assumptions lead to slight underestimation of true lengths.

Standard Fixation Procedure:

The data from the sequences of serial sections through arrays of cortical microtubules in *Azolla pinnata* root tip cells and the calculated average lengths are shown in Table 3.2. There is considerable deviation from the mean in each category, but the average lengths, in general, are approximately 2 to 4 µm, irrespective of the type of array. Cortical microtubules in root tips of *Zea mays* and *Impatiens balsamina* were also serially sectioned and the average microtubule lengths calculated. As in *Azolla*, the average lengths are short relative to the circumference of the cell: 3-4 µm in *Zea Mays* and 5-6 µm in *Impatiens balsamina* (Table 3.3). In all three plants the average microtubule length was approximately one eighth of the circumference of the cells that were examined.
Alternative Fixation Procedures:

The duration of glutaraldehyde pre-fixation (see Table 3.2) was varied when preparing the material described in the preceding section, and it was clear that these variations had no major effect on the results. In the course of the work, however, it was reported that a greater microtubule profile length could be observed in HeLa cells if the fixative included co-factors of the type employed when polymerising tubulin in vitro (Luftig et al., 1976, 1977). Accordingly, sequences of serial sections were cut through cortical microtubule arrays in *Azolla* cells which had been fixed in 2.5% glutaraldehyde in a solution containing 1mM guanosine triphosphate (GTP), 1mM magnesium sulphate, 2mM ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid and 100mM piperazine-N,N'-bis-(2-ethane sulphonic acid) buffer, pH 6.9. In other experiments the GTP was omitted.

The general ultrastructure of the cells, and more specifically, of the microtubule arrays, did not differ from that seen after conventional fixation in phosphate-buffered glutaraldehyde. A reconstruction of an interphase array in tissue fixed in the complete "polymerisation medium" is shown in Fig. 3.28 and the average microtubule lengths are listed in Table 3.4. The mean value for the inter-phase microtubules is slightly longer in the material fixed in the complete polymerisation medium.
than in conventionally prepared roots, but the difference is not significant. The microtubules are still short relative to the cell circumference. The mean value for the average microtubule length in roots fixed in the polymerisation medium lacking GTP is less than that in complete medium (but non-significantly) and in fact is very close to that obtained in the conventional glutaraldehyde/phosphate-fixed material. None of the 181 terminations observed after fixation in polymerisation medium was C-shaped (Table 3.6).

A root fixed in polymerisation medium provided another example of abrupt changes in the numbers of microtubules per section due to the presence of lines of terminations. The sequence, only 16 sections long, cut through an initially highly stacked pre-prophase band, the reconstruction of which is shown in Fig. 3.18. Three bands of terminations are evident, all parallel to the plane of sectioning and giving rise overall to significant deviation from the Poisson distribution. The microtubule terminations in the other sequences of sections of material fixed in this way were randomly distributed according to both Poisson and pattern analyses.

3.3.6 Complete Microtubules

As is evident from the microtubule maps, many of the sequences of serial sections included
a number of complete microtubules. An example is included in Fig. 3.10. Table 3.5 lists the numbers of complete microtubules in four length categories, expressed in each case as a percentage of the total number of microtubules present in the map. Because terminations have been arbitrarily taken to occur half way through the section, the complete lengths cited in the Table are one section thickness less than the combined thickness of the sections in which the microtubule was seen. The Table includes only complete microtubules up to 1.4µm in length, although longer ones did occur in the longer sequences. The advantage of expressing the numbers of complete microtubules in this way is that sequences of similar total thickness can be compared. For example, in a comparison of conventional fixation versus fixation in polymerisation medium, no differences emerge. On the other hand, some of the treatments to be described below had marked effects on the length distribution of the microtubules.

3.3.7 Treatments which can cause Depolymerisation of Microtubules

*Low Temperature:* The cold treatments employed in this work did not lead to the complete disappearance of microtubule arrays in the root tip cells. 22.4% of all terminations found in material fixed in the cold without any recovery time were C-shaped - thirteen
times the frequency seen in control tissues. In one sequence of sections 2 C-shaped terminations pointed in one direction and 10 in the other; in another sequence 2 pointed one way and 1 in the other. The distribution of terminations was random in two sequences of sections of low temperature treated roots (e.g. Fig. 3.30) but the one illustrated in Fig. 3.29 was non-random according to the Poisson and the pattern analyses.

Calculated average lengths for the microtubules (Table 3.7A) reveals a diminution to about half the average length found in untreated tissues (Figs. 3.29, 3.30) and a rapid (15 minutes) recovery to the normal range of lengths (Fig. 3.31). The proportion of short microtubules was only slightly greater than in controls (Table 3.5B).

High Pressure: The effect of high pressure is not straightforward. After a 30 minute treatment at 6,000 psi the average length was 5.2μm (Table 3.7B).

A reconstruction of a pre-prophase band in a root fixed immediately after 15 minutes at 14,000 psi is shown in Fig. 3.32. Poisson and pattern analyses both indicate that the terminations are non-randomly distributed in this 55 section sequence. The proportion of short complete microtubules is large (Table 3.5B), 28% of the microtubules are less than 0.35 m in length, compared with 14% and 0% in this size class in the two 50-section long sequences.
in the controls. A 33-section sequence through an interphase array following 16,000 psi treatment (Fig. 3.33) shows the same situation: 16% of the microtubules in the array are less than 0.35 µm in length; there are no complete microtubules of this length in either the 33- or the 30-section sequences in the control roots. The proportion of C-shaped terminations was as in controls, and only one sequence had more than one present. In it both C-shapes pointed in the same direction.

**Colchicine:** Microtubules were present in some *Azolla* root tip cells even after 2-5 hours in 5 x 10^{-3}M colchicine solution (Figs. 3.34-3.36). In roots fixed immediately after colchicine treatment the proportion of C-shaped terminations was 3.6% (Table 3.6). Two sequences had two C-shaped terminations, and in each the two C-shapes pointed in opposite directions. The percentage of short complete microtubules was raised relative to the controls (Table 3.5B), and the average microtubule lengths were approximately one third to one half those in untreated roots. After a one hour recovery period, the average length was even shorter (Table 3.7C). In three of the six sequences the microtubule terminations were non-randomly distributed.

3.3.8 Treatment with Deuterium Oxide

Exposure of *Azolla* roots to D_{2}O for 5 hours
produced microtubule arrays which contained large numbers of short microtubules. 23% of the interphase microtubules in the sequence shown in Fig. 3.37 and 25% in another sequence 50 sections long, are less than 0.35 \( \mu \)m in length (Table 3.5C), 37% and 38% being less than 0.7 \( \mu \)m. The latter values can be compared with the 4% - 18% in control sequences containing similar numbers of sections. The average microtubule length was approximately half that in controls (Table 3.8). The frequency of C-shaped terminations was 0.9% (Table 3.6). Only one sequence contained more than one, and in it all 3 C-shapes pointed in the same direction. Short microtubules appear in response to D\(_2\)O treatment amongst those that are found over developing xylem thickenings (Fig. 3.38); microtubules may in addition be initiated between the thickenings, where their frequency would normally be low (cf Fig. 3.13).

After 18 hours in D\(_2\)O (Fig. 3.39) the average length was nearly as in controls (Table 3.8), as was the length distribution (Table 3.5C). The frequency of C-shaped terminations was zero (Table 3.6).

3.4 DISCUSSION

The major result reported here is that most cortical microtubules in plant cells are short relative to the dimensions of the cell circumference. Microtubule arrays
may extend over large or small expanses of cell cortex, but the continuity of an array is merely statistical, the constituent microtubules being present as overlapping units of varying but limited length, on average one cross sectional profile in every 18 representing a terminating microtubule. Before discussing the implications of this observation, the validity of the methods used must be examined.

3.4.1 Methods

No direct check on the efficacy of glutaraldehyde fixation with respect to cortical microtubules has yet been devised. Attempts to use measurements of birefringence in mitotic spindles as a non-destructive method to assess microtubule densities in vitro, followed by comparison with counts made after fixation, would not be suitable for the plant cell cortex where any birefringence due to the microtubules would be masked by that of the cell wall. Glutaraldehyde does, however, fix microtubules that have been polymerised in vitro, and affords protection from alterations to their length (Luftig et al., 1977). It also preserves rings, sheets, and tubules in the same proportions as observed in negatively-stained preparations of polymerising mixtures, and it prevents free tubulin from polymerising (Kirschner et al., 1975).

It was found that the duration of the glutaraldehyde
fixation step can be altered between 10 minutes and 17 hours with no marked effects upon microtubule length (Table 3.2). Dispositions that might be interpreted as being due to an originally intact microtubule fracturing and the ends so formed moving apart are rare in the reconstructions: one example is in section 41 of Fig. 3.23A. The existence of cell-specific features in the back-to-back arrays of Figs. 3.23A and B also argues against major mechanical damage during specimen processing. Reports (Luftig et al., 1976, 1977) that relatively slow entry of glutaraldehyde permits the buffer to alter the degree of polymerisation of tubulin within HeLa cells appeared in the course of the present work. In Azolla roots, unlike HeLa cells, the microtubule lengths are essentially the same whether the glutaraldehyde is dissolved in phosphate buffer or in polymerisation medium with or without GTP.

The method of tracking microtubules from section to section has been used in a number of situations (Heath, 1974b; Heath and Heath, 1976; Warren, 1974), with considerable difficulty in the case of very complex arrays such as large mitotic spindles (Jensen and Bajer, 1973; Lambert and Bajer, 1972; Manton et al., 1969; McIntosh, Cande, Snyder, 1975; McIntosh, Cande, Snyder and Vanderslice, 1975). If microtubules longer than those detected by the tracking procedure were consistently found in planes of section
that show their longitudinal aspect (e.g. Figs. 3.16, 3.19, 3.21, 3.24), then doubt would be cast on the methods used here. In fact, the longest segment of cortical microtubule found in a collection of several thousand micrographs of *Azolla* root tips measured 2.5µm. All papers on cortical microtubules listed in a recent review (Hepler and Palevitz, 1974) have been inspected, and again, no microtubules longer than 1-2µm were found in the published micrographs, with the exception of two examples, at 3.9 and 3.6µm, in freeze-fractured pea root tip cells (Northcote and Lewis, 1968). Longer profiles would be expected in freeze-fractured material as a fracture plane can follow a curved cell cortex whereas a planar ultra-thin section cannot. There may, however, be an additional factor, for the pea roots in question were soaked, without prefixation, for 1-10 days in 20% glycerol. Glycerol is now known to support polymerisation of tubulin (Shelanski *et al.*, 1973), binding to and allowing otherwise inactive tubulin to assemble into microtubules (Detrich *et al.*, 1976). Thus there is no conflict between the longitudinal views and the results obtained by tracking and this, together with the ability to detect inter-specific and inter-treatment differences in microtubule lengths gives added confidence that the fixation and tracking procedures used here are valid, at least for the relatively simple arrays found in the plant cell cortex.
3.4.2 Arrays of Overlapping Constituent Microtubules

Cortical arrays in plant cells are not the only examples of systems composed of overlapping microtubules. They occur in myogenic cells (Warren, 1974) and in a variety of mitotic spindles: in non-kinetochore fibres in *Haemanthus* endosperm (Jensen and Bajer, 1973; Lambert and Bajer, 1972); in an insect spermatocyte (Fuge, 1974), where <1% of the tubules were traced and lengths in the range 1.0 -5.5µm found; in the spindle of *Saccharomyces* (Peterson and Ris, 1976); in mammalian cells in tissue culture (McIntosh, Cande and Snyder, 1975; McIntosh, Cande, Snyder and Vanderslice, 1975); and in the fungi *Thraustotheca* (Heath, 1974b) and *Uromyces* (Heath and Heath, 1976). The formulae used in the present work can be applied to two of the published sets of data. In myogenic cells (Warren, 1974) data for one set of serial sections yield a calculated average microtubule length of 27µm in a cell approximately 150µm long, with the distribution of the observed terminations conforming to a Poisson distribution. In *Uromyces* (Heath and Heath, 1976) a 22-section sequence contained complete microtubules between 1 and 15 section thicknesses in length, and the formula gives an average length of 2µm.

That cortical arrays of microtubules in plant cells consist of overlapping microtubules
provides an explanation for the disparity in numbers that has sometimes been recorded for opposite sides of the same cell, in wheat\textsuperscript{†} and \textit{Azolla} (present work) root tip cells, cells of \textit{Sphagnum} "leaflets" (Schnepf, 1973; Schnepf \textit{et al.}, 1976), and in hypocotyl cells (Sawhney and Srivastava, 1975). In both \textit{Azolla} (Section 4.3.2) and \textit{Sphagnum} (Schnepf \textit{et al.}, 1976) the numbers are greater along the younger wall. Such occurrences would not be possible if the microtubules were in the form of continuous hoops or spirals, and their implication is that the density of the array can alter across the face of the cell. In at least some cases, particularly in pre-prophase bands (Figs. 3.15, 3.18), it is very probable that the density differences arise because the development of the array is incomplete or because it is breaking down progressively from one side of the cell.

The lengths of the complete microtubules in the sequences of sections varied widely, and it is important to know whether there might be a proportion of very long representatives, which might approach the dimensions of the "hoops" that are referred to so often in the current literature (see Section 3.1).

Complete microtubules contained within the longer sequences of sections were classified

\textsuperscript{†}O'Brien and Maynard, personal communication.
according to length and cumulative distribution curves plotted in the hope that predictive formulae could be fitted. No consistent trends were found and it was therefore not possible to predict the overall frequency distributions of microtubule lengths from the incomplete data that are available. Some indication is, however, gained from the summation in Fig. 3.40, where the points that are plotted represent, for each sequence of serial sections, the number of microtubules that passed right through the sequence with neither termination included, normalised by expressing it as a fraction of the average microtubule number in the array; this fraction is plotted against the number of sections in the sequence. As would be expected, the value of the fraction approaches unity as the number of sections per sequence is reduced, and it diminishes as the number of sections increases, reaching zero in two of the longest sequences. Values for all fixation regimes and all categories of microtubule array in untreated roots were pooled before using the least squares method to fit the exponential decay curve shown in Fig. 3.40. The results may be interpreted as showing an exponentially decreasing probability of finding microtubules of length equal to or greater than the combined section thicknesses, as the number of sections per sequence is increased. Extrapolation of the curve of best fit suggests that if it were possible in practice
to follow the whole circumference of a 10 μm
diameter cell by serial sectioning, the probability
of finding a microtubule of length equal to the
total circumference would be 8 x 10^-6. The avail­
able data thus point strongly to the absence of
microtubules of length comparable to the cell
circumference.

3.4.3 Perturbations of the Arrays

The data on treatments which in other
systems alter the stability of the tubulin: microtubule
equilibrium in favour of either assembly or dis­
assembly are limited because the sample sizes
obtained through the use of serial sectioning are
extremely small. Further, particularly in the case
of the depolymerising treatments - exposure to low
temperature, high pressure, and colchicine - the
sequences of sections are highly selected because
the majority of cells had lost a considerable pro­
portion of their cortical microtubules. It is not
known whether such losses might be selective, as in
other systems (Brinkley and Cartwright, 1975; Salmon
et al., 1976). The microtubule maps (Figs. 3.28-3.36)
and data (Tables 3.5B and 3.7) presented here
therefore represent either surviving or recovering
areas of cell cortex, examined in the hope that the
form of the microtubules might give some insights
into the dynamics of the arrays.
The depolymerising treatments fall into two categories, though all three reduced the average length (Table 3.7). Colchicine and high pressures gave rise to arrays with greatly augmented proportions of short microtubules (Table 3.5) without, however, producing large increases in the proportion of C-shaped terminations (Table 3.6). Low temperature, on the other hand, had little effect on the length-distribution, but produced very high frequencies of C-shapes. In this case, an interpretation that is consistent with the data is that the microtubules had (on average) shortened, many of them opening out to give the C-profile; the short microtubules initially present had disappeared and were replaced by remnants of longer ones, the average length thus falling. It is not ruled out, however, that some C-shapes might be generated in the cold without accompanying disassembly. The former pattern is suggestive of fragmentation, presumably in addition to disassembly or alternatively of early stages in recovery when the population contained short but growing microtubules. Recent evidence suggests that low temperatures and high pressures induce a conformation change of the tubulin subunits thus influencing the microtubule nucleation process (Engelborghs et al., 1976). Microtubule shortening during exposure to low temperature or early stages of recovery following high pressure treatment would be consistent with this
mechanism. *Azolla*, like most plants (Hart and Sabnis, 1976), requires higher concentrations of colchicine for disassembly than do animal cells. As in the fungus *Saprolegnia* (Heath, 1975b), a considerable resistance to high pressure was found. Pressures that affect sea urchin gastrulae (Tilney and Gibbins, 1968), HeLa cell spindles (Salmon et al., 1976), the unicell *Ochromonas* (Brown and Bouck, 1973), heliozoan axopodia (Tilney, 1971; Ockleford and Tucker, 1973) and tubulin in vitro (Salmon, 1975) were relatively innocuous to *Azolla*.

In general, D$_2$O is reported to stabilise microtubule arrays and enhance microtubule formation from available pools of tubulin (Inoué and Ritter, 1975; Stephens, 1973). For plant cells in interphase, no effects were found in wheat root tips (Burgess and Northcote, 1969) and rye leaves (Waber and Sakai, 1975), yet in *Sphagnum* leaf cells at the same stage of the cell cycle there was a 25% increase in the number of microtubules (Schnepf et al., 1976). For pre-prophase bands, a marked increase in microtubule numbers was claimed for wheat roots (Burgess and Northcote, 1969), yet in *Sphagnum* the band appeared more transient and indistinct (Schnepf et al., 1976). Observations of pre-prophase bands in untreated *Azolla* roots show great variation in microtubule numbers (Chapter 5), presumably reflecting developmental changes, and the map shown in Fig. 3.18 serves to emphasise that the number of microtubules
per section can be a poor criterion for this type of experiment. More reliance can, however, be placed on observations of microtubule length within a given array. A 5 hour treatment in D₂O skewed the length distribution strongly towards short microtubules as compared with controls (Table 3.5). This applied to all three categories of array and a likely interpretation is that additional tubules were generated which, being short, lowered the overall average length (Table 3.8). The proportion of C-shaped terminations was lower than in controls and there was no general diminution in microtubule numbers throughout the root tip such as was observed in the depolymerising treatments. After 18 hours treatment the length distribution and the average length approximated to the control situation, and in the absence of firm information on total numbers per cell or per unit length of wall the only valid interpretation of this seeming return to normality is that the response to D₂O does not involve enhancement of assembly onto existing microtubules (which would have increased the average length), but rather the generation of new ones. The evidence of Fig. 3.38 and the finding that additional microtubules appear in the centre of the cell (Schnepf et al., 1976) suggest that under the influence of D₂O at least some of the new microtubules can be generated in positions where they would not normally occur.
Treatments which should favour polymerisation (presence of GTP, D$_2$O) diminished the proportion of terminations with C-shapes, whereas low temperature and (to a lesser extent) colchicine increased this proportion. These data support the view that the C-shape is associated with disassembly (Roth and Shigenaka, 1970), notwithstanding the claims that it can also occur at growing terminations in vivo (Behnke, 1967) and in vitro (Bryan, 1976) [for full discussion see Cohen and Gottlieb, 1971; Jensen and Bajer, 1973; Lambert and Bajer, 1972].

C-shaped profiles were not restricted to the ends of the microtubules, even in untreated material, and low temperatures enhanced the proportion of sub-terminal C-shapes.

Of the 325 complete microtubules that were mapped, 15 had visible manifestation of polarity, viz, a C-shaped profile at one end but not at the other. No microtubules with C-shapes at both ends were found. An obvious interpretation of this morphological polarity is that the microtubules grow or disassemble preferentially at one end, as has been observed in vivo (Rosenbaum et al., 1969; Ockleford and Tucker, 1973; Osborn and Weber, 1976) and in vitro (see Dentler, 1974; Snyder and McIntosh, 1976). It is therefore of great interest that C-shapes are patterned in the microtubule maps. When serial sections are examined, the C-shapes point in the same direction. The observed numbers of C-shapes
were as follows (expressed in the form of the number of C-shapes pointing one way: number pointing the opposite way) 0:2, 0:4, 0:6 (controls); 0:3 (5h D$_2$O); 0:2 (high pressure); 2:10, 1:2 (low temperature); and 1:1, 1:1 (colchicine). The observations on colchicine-affected arrays are few, but suggest that the microtubules in them, although still polar as individuals, do not share a common polarity. In the controls and D$_2$O treatment, at least those microtubules that have C-shaped terminations share a common morphological directionality. It is not known whether this sharing extends to the majority of the microtubules (those lacking C-shaped terminations), nor is it known whether all faces, or indeed all parts of faces, of a cell share the same directionality. The cell cortex could be subdivided into domains that differ from one another but within which directionality is shared.

3.4.4 Development of Cortical Arrays

In undertaking the present work, one expectation was that evidence for microtubule organising structures might emerge. In fact the great majority of microtubule terminations are not patterned, with the exception of some clustering (e.g. Fig. 3.20). Precedents for linear MTOCs which generate microtubules at right angles to their long axis do exist in the algae (Bouck and Brown, 1973; Brown and Bouck, 1973), but no ultrastructural evidence
for any equivalent of these rhizoplast fibres was found here. The lines of terminations may well be significant, particularly the two that lay back-to-back in neighbouring cells, in or close to the direction of cell elongation (Fig. 3.23). They can, however, hardly be indicative of MTOCs unless these are so few in number that the sample sequences of sections rarely included them, or unless some form of propagation and turnover of the population of microtubules obliterates a patterned origin that is conspicuous only transiently in an earlier stage of the cell cycle. It must be emphasised that the sample sizes are not only small relative to the surface area of the cell, but also selective: because of the practical problems of tracking microtubules, they did not penetrate into any cell corners, which therefore remain as possible sites for MTOCs.

The majority of cortical microtubules, short or long, lie parallel to and at varying distances from the plasma membrane, this being especially evident in highly stacked pre-prophase bands. Detailed examination of the serial sections shows that microtubules, even very short ones, do not bend towards and terminate "in or on the plasma membrane", as suggested for Allium root tip pre-prophase bands (Packard and Stack, 1976). The source and mechanism of placement of any initiator molecules that may be required (see Bryan, 1976; Snyder and McIntosh, 1976)
are therefore unknown. It should be pointed out that the microtubules are not necessarily assembled where they are seen. Neither the literature nor the present results preclude the concept of an assembly process that is followed by mobility of microtubules within the arrays. Once initiated, the ultimate length of the microtubules could be governed by the balance between the numbers of free tubulin molecules and the numbers of competing assembly sites, as demonstrated in vitro in experiments where a given quantity of tubulin can be used to make many short microtubules or fewer longer ones (Bryan, 1976; Slobada et al., 1976). Further experiments using D$_2$O which, as described above, seems to induce additional microtubules rather than elongate existing ones, might be helpful in this context.

Some estimates of microtubule growth rates are available. In plant material, microtubule growth keeps pace with the 1-2µm minute$^{-1}$ elongation rate of root hairs (Newcomb and Bonnett, 1965). Values of up to 1.4µm minute$^{-1}$ for in vitro growth are reported dependent on the tubulin concentration of the polyanion (Bryan, 1976). The rate of 1.5µm minute$^{-1}$ claimed for reassembling mitotic spindles (Goode, 1973) may be an overestimate in that it rests on the assumption that the spindle fibres, which were measured by optical microscopy, consist of continuous rather than overlapping microtubules,
with single rather than multiple growing points. The *Azolla* roots used here were elongating and data on the interpolation of additional microtubules along the cell walls detailed in Section 4.3.2, suggest that their growth rates approach the above values. It is not known, whether, or how, turnover of the components of cortical microtubule arrays is accomplished. At one extreme, entire microtubules could disassemble and be replaced at the growth rates suggested above, in which case the lifetime of a 2-4µm long microtubule might be only a few minutes. The low frequency (1.7%) of C-shaped terminations militates against this view. Alternatively, individual microtubules, once formed, might be stabilised through having reached an equilibrium between addition and loss of tubulin, or by becoming cross-bridged to adjacent structures.

Bridges occur between adjacent microtubules and between microtubules and membranes in *Azolla* as in other plant cells (Hepler and Palevitz, 1974). Periodicity of cross-bridging is shown clearly in Fig. 3.4, but the resolution is insufficient to show whether it conforms with the generalisation that the periodicities of arms and bridges are multiples of the 8nm axial periodicity defined by the microtubule structural unit, as in the 24nm axial period of the dynein arms along the outer doublet tubules of flagella (McIntosh, 1974). The present observation of periodic cross-bridging
in arrays of plant cortical microtubules is of potential significance with respect to possible functions, be they in microtubule assembly, in stabilising and orienting the microtubules, in stabilising domains or molecules in or on the plasma membrane, or in mediating motility phenomena.

3.4.5 Microtubules and Microfibrils

Much of the preceding discussion has centred on the properties of the extensive microtubule arrays that lie against the longitudinal walls of elongating *Azolla* root tip cells, where the microtubules and the cellulose microfibrils\(^*\) share a predominantly transverse orientation, just as in other species (see Section 1.4.3). However, deposition of cellulose can also be restricted to precisely localised areas of the cell surface, as at pits (Robards and Humpherson, 1967) or the thickenings of stomatal pores (Palevitz and Hepler, 1976). Whereas it is difficult to envisage how arrays of long "hoop-like" microtubules could be locally differentiated in order to participate in such events, the present observation that cortical arrays consist of short overlapping components does at least allow the concept of microtubule participation to be applied to both large and small expanses of wall: the only difference in principle is the degree to which the array of overlapping microtubules is propagated around the cell cortex.

\(^*\) Polarised light microscope observations by Dr. P.B. Green, Stanford University, personal communication.
At both pits and stomatal pores the microtubules and wall microfibrils are co-aligned, emphasising once again the major problems of how microtubule arrays become localised and oriented. It may be that the systems that control the formation of extensive as compared to spatially restricted arrays are considerably different.

The observations presented here have no direct bearing on the possible role of cortical microtubules in orienting the deposition of cellulose microfibrils. However, the view that the cortical microtubules represent "relatively rigid tracks along which other cellular components [such as cellulose synthetase complexes projecting inwards through the plasma membrane to the microtubules] might move" (Heath, 1974a) is now unattractive because the "tracks" are seen to be too short. Guidance of cellulose synthetase complexes by means of microtubule movements generated by inter-tubule sliding or polarised assembly-disassembly is not ruled out, and neither is indirect guidance by the production of shearing forces in the membrane by motility-generating molecules associated with the cortical microtubules (Hepler and Palevitz, 1974). Indeed, the latter suggestions receive a measure of support from the detection of polarity in the cortical arrays.
TABLE 3.1  Average section increments for a Reichert OMU3 ultramicrotome cutting Spurr's resin blocks.

<table>
<thead>
<tr>
<th>Interference colour</th>
<th>θ</th>
<th>N</th>
<th>Average section increment (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver - grey</td>
<td>70°</td>
<td>150</td>
<td>61</td>
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<tr>
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<td>75°</td>
<td>100</td>
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<td>60°</td>
<td>200</td>
<td>67</td>
</tr>
<tr>
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<td>170</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>50°</td>
<td>270</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>50°</td>
<td>250</td>
<td>79</td>
</tr>
<tr>
<td>Silver - light gold</td>
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<td>165</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>55°</td>
<td>155</td>
<td>84</td>
</tr>
</tbody>
</table>


### Table 3.2

**Average lengths of cortical microtubules in Azolla root tip cells**

<table>
<thead>
<tr>
<th>Type of microtubule array</th>
<th>Number of sections in sequence (x0.07=na, µm)</th>
<th>Average number of microtubules per section (N)</th>
<th>Number of terminations in sequence (T)</th>
<th>Calculated average length (L, µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At xylem thickenings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9*</td>
<td>6</td>
<td>2</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>9*</td>
<td>9</td>
<td>4</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>9*</td>
<td>9</td>
<td>7</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>9*</td>
<td>13</td>
<td>10</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>12*</td>
<td>14</td>
<td>12</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>12*</td>
<td>16</td>
<td>8</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>mean</strong> 2.5±0.97</td>
<td></td>
</tr>
<tr>
<td>Pre-prophase band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41*</td>
<td>16</td>
<td>28</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>21‡</td>
<td>37</td>
<td>44</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>22‡</td>
<td>51</td>
<td>80</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>33§</td>
<td>47</td>
<td>109</td>
<td>2.0</td>
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<td></td>
<td></td>
<td></td>
<td><strong>mean</strong> 2.4±0.57</td>
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</tr>
<tr>
<td>Interphase</td>
<td></td>
<td></td>
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<tr>
<td>14§</td>
<td>11</td>
<td>5</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>14§</td>
<td>11</td>
<td>4</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
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<td>9</td>
<td>9</td>
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<tr>
<td>12*</td>
<td>15</td>
<td>9</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>23§</td>
<td>8</td>
<td>16</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>23§</td>
<td>11</td>
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<td>1.8</td>
<td></td>
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<td>20</td>
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<td></td>
<td></td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>76‡</td>
<td>10</td>
<td>57</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>30**</td>
<td>27</td>
<td>22</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>74‡</td>
<td>16</td>
<td>66</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>40*</td>
<td>30</td>
<td>42</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>90**</td>
<td>16</td>
<td>72</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>mean</strong> 3.1±1.23</td>
<td></td>
</tr>
</tbody>
</table>

Duration of fixation in glutaraldehyde: * 4 hours; ‡ 8 hours; § 10-15 minutes; || 2 hours; * 6 hours; ** 17 hours. Each line in the Table represents a different sequence of serial sections. The first three columns of data give the quantities needed to calculate the average microtubule length (L) using the formula L=2Na/T. Individual and mean values for each category of microtubule array are given in the last column.
### Average lengths of cortical microtubules in *Zea* and *Impatiens* root tip cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of microtubule array</th>
<th>Number of sections in sequence</th>
<th>Average number of microtubules per section</th>
<th>Number of terminations in sequence</th>
<th>Calculated average microtubule length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zea mays</em></td>
<td>Pre-prophase band</td>
<td>22</td>
<td>41</td>
<td>44</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>18</td>
<td>10</td>
<td>10</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>14</td>
<td>8</td>
<td>4.7</td>
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<tr>
<td></td>
<td></td>
<td>18</td>
<td>18</td>
<td>12</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean</td>
<td>3.7±1.05</td>
</tr>
<tr>
<td><em>Impatiens balsamina</em></td>
<td>Interphase</td>
<td>30</td>
<td>11</td>
<td>10</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>25</td>
<td>12</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>20</td>
<td>10</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mean</td>
<td>5.6±1.26</td>
</tr>
</tbody>
</table>
### Table 3.4

**Average lengths of cortical microtubules in *Azolla* following fixation in a microtubule-polymerisation medium**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of microtubule array</th>
<th>Number of sections in sequence</th>
<th>Average number of microtubules per section</th>
<th>Number of terminations in sequence</th>
<th>Calculated average microtubule length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete polymerisation</td>
<td>At xylem thickenings</td>
<td>19</td>
<td>17</td>
<td>14</td>
<td>3.2</td>
</tr>
<tr>
<td>Polymerisation medium</td>
<td>Pre-prophase band</td>
<td>16</td>
<td>46</td>
<td>98</td>
<td>1.0</td>
</tr>
<tr>
<td>Interphase</td>
<td></td>
<td>6</td>
<td>11</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
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<td>8</td>
<td>10</td>
<td>4</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>15</td>
<td>4</td>
<td>3.2</td>
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<td>14</td>
<td>8</td>
<td>2</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>18</td>
<td>4</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>19</td>
<td>9</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>11</td>
<td>7</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39</td>
<td>19</td>
<td>35</td>
<td>3.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>3.8±1.8</strong></td>
</tr>
</tbody>
</table>

| Polymerisation medium minus G.T.P. | Interphase | 8 | 12 | 6  | 2.2 |
|                                   | medium     | 13 | 8  | 7  | 2.1 |
|                                   | minus G.T.P.| 10 | 18 | 4  | 6.2 |
|                                   |            | 13 | 22 | 5  | 2.7 |
| Mean                               |            |    |    |    | **3.3±1.95** |
TABLE 3.5

Percentages of complete microtubules in cortical microtubule arrays in Azolla root tip cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of microtubule array</th>
<th>Number of sections in sequence</th>
<th>Total number of microtubules</th>
<th>Complete microtubules, as % of total No., in different length classes (μm):</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Glutaraldehyde in 0.025M phosphate</td>
<td>Interphase</td>
<td>90</td>
<td>52</td>
<td>2 2 2 2</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>76</td>
<td>39</td>
<td>13 10 0 8</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>74</td>
<td>51</td>
<td>2 12 2 8</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>50</td>
<td>24</td>
<td>14 4</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>50</td>
<td>27</td>
<td>0 4 4</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>41</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>40</td>
<td>52</td>
<td>4 7</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>33</td>
<td>109</td>
<td>0 6 4 1</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>30</td>
<td>38</td>
<td>0 3</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>22</td>
<td>95</td>
<td>2 2</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>21</td>
<td>58</td>
<td>7 3 2</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>20</td>
<td>33</td>
<td>6 3</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>20</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde in complete polymerisation medium</td>
<td>Interphase</td>
<td>39</td>
<td>41</td>
<td>2 5</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>21</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>B. Low temperature</td>
<td>Pre-prophase</td>
<td>31</td>
<td>40</td>
<td>8 5 5</td>
</tr>
<tr>
<td>0°C 15 min</td>
<td>Interphase</td>
<td>26</td>
<td>18</td>
<td>4 0 4 4</td>
</tr>
<tr>
<td>1.6°C 4 h</td>
<td>Interphase</td>
<td>22</td>
<td>27</td>
<td>0 0 5</td>
</tr>
<tr>
<td>2°C 15 min + 15 min recovery</td>
<td>Interphase</td>
<td>55</td>
<td>71</td>
<td>28 6 4 3</td>
</tr>
<tr>
<td>High pressure:</td>
<td>Interphase</td>
<td>33</td>
<td>25</td>
<td>16 4 8</td>
</tr>
<tr>
<td>14,000 psi</td>
<td>Interphase</td>
<td>19</td>
<td>48</td>
<td>15 14</td>
</tr>
<tr>
<td>16,000 psi</td>
<td>Interphase</td>
<td>29</td>
<td>19</td>
<td>0 11 11</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Interphase</td>
<td>39</td>
<td>26</td>
<td>4 4</td>
</tr>
<tr>
<td>2 h</td>
<td>Interphase</td>
<td>39</td>
<td>26</td>
<td>4 4</td>
</tr>
<tr>
<td>3 h</td>
<td>Interphase</td>
<td>31</td>
<td>27</td>
<td>11 8 4</td>
</tr>
<tr>
<td>5 h</td>
<td>Interphase</td>
<td>18</td>
<td>31</td>
<td>16 3</td>
</tr>
<tr>
<td>2 h + 1 h recovery</td>
<td>Xylem</td>
<td>29</td>
<td>38</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>50</td>
<td>194</td>
<td>25 13 7 3</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>56</td>
<td>66</td>
<td>23 14 2</td>
</tr>
<tr>
<td>C. Deuterium oxide</td>
<td>Interphase</td>
<td>34</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>5 h</td>
<td>Interphase</td>
<td>35</td>
<td>23</td>
<td>4 4</td>
</tr>
</tbody>
</table>
### Table 3.6 Percentages of C-shaped terminations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of terminations observed</th>
<th>% of C-shaped terminations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>706</td>
<td>1.7</td>
</tr>
<tr>
<td>Polymerisation medium</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Low temperature</td>
<td>67</td>
<td>22.4</td>
</tr>
<tr>
<td>High pressure</td>
<td>234</td>
<td>1.7</td>
</tr>
<tr>
<td>Colchicine</td>
<td>139</td>
<td>3.6</td>
</tr>
<tr>
<td>5 h D₂O</td>
<td>445</td>
<td>0.9</td>
</tr>
<tr>
<td>18 h D₂O</td>
<td>87</td>
<td>0</td>
</tr>
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</table>
### Table 3.7

Average lengths of cortical microtubules in Azolla root tip cells following depolymerization treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of microtubule array</th>
<th>Number of sections in sequence</th>
<th>Average number of microtubules per section</th>
<th>Number of terminations in sequence</th>
<th>Calculated average microtubule length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Low temperature</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°C 15 min</td>
<td>Interphase</td>
<td>31</td>
<td>19</td>
<td>48</td>
<td>1.7</td>
</tr>
<tr>
<td>1.6°C 4 h</td>
<td></td>
<td>26</td>
<td>8</td>
<td>19</td>
<td>1.5</td>
</tr>
<tr>
<td>2°C 15 min + 15 min recovery</td>
<td></td>
<td>22</td>
<td>17</td>
<td>18</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>B. High pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,000 psi. 30 min</td>
<td>Interphase</td>
<td>14</td>
<td>21</td>
<td>8</td>
<td>5.2</td>
</tr>
<tr>
<td>6,000 psi. + 2 hr recovery</td>
<td>Pre-prophase</td>
<td>14</td>
<td>74</td>
<td>95</td>
<td>1.5</td>
</tr>
<tr>
<td>14,000 psi. 15 min</td>
<td></td>
<td>55</td>
<td>17</td>
<td>108</td>
<td>1.2</td>
</tr>
<tr>
<td>16,000 psi. 15 min</td>
<td>Interphase</td>
<td>33</td>
<td>9</td>
<td>31</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>C. Colchicine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr</td>
<td>Interphase</td>
<td>19</td>
<td>15</td>
<td>53</td>
<td>0.8</td>
</tr>
<tr>
<td>2 hr + 1 hr recovery</td>
<td></td>
<td>29</td>
<td>11</td>
<td>20</td>
<td>1.9</td>
</tr>
<tr>
<td>3 hr</td>
<td></td>
<td>18</td>
<td>8</td>
<td>36</td>
<td>0.6</td>
</tr>
<tr>
<td>5 hr</td>
<td></td>
<td>39</td>
<td>14</td>
<td>25</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31</td>
<td>6</td>
<td>33</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 3.8

Average lengths of cortical microtubules in *Azolla* root tip cells following deuterium oxide treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Type of microtubule array</th>
<th>Number of sections in sequence</th>
<th>Average number of microtubules per section</th>
<th>Number of terminations in sequence</th>
<th>Calculated average microtubule length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 h D₂O</td>
<td>Xylem</td>
<td>11</td>
<td>7</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>16</td>
<td>17</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>15</td>
<td>38</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Pre-prophase</td>
<td>50</td>
<td>50</td>
<td>293</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>13</td>
<td>9</td>
<td>21</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>56</td>
<td>16</td>
<td>96</td>
<td>1.3</td>
</tr>
<tr>
<td>18 h D₂O</td>
<td>Pre-prophase</td>
<td>8</td>
<td>46</td>
<td>24</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Interphase</td>
<td>13</td>
<td>10</td>
<td>7</td>
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FIG. 3.1 Diagrams showing a block trimmed with slopes of known angle before (a) and after (b) taking off a sequence of serial sections, and the relationship between the measurements of the block face and the average section increment.

FIG. 3.2 Microtubules, length L, lie adjacent to the perimeter of a cell circumference, A. The sample arc, a, is equal to the thickness of the combined serial sections.
Average section increment = \( \frac{(CD - AB)}{2N\tan \theta} \)

Fig. 3.1

Fig. 3.2
FIGS. 3.3-3.9 The tracking of individual microtubules: microtubule cross-bridges.

FIGS. 3.3, 3.4 Two adjacent serial sections through a pre-prophase band of microtubules illustrate the difficulty of tracking microtubules in longitudinal section. Microtubules that have been sectioned transversely can, however, be followed from one section into the next (see boxes). Periodic bridging between adjacent longitudinally-sectioned microtubules is evident in the section in Fig. 3.4 (arrows). X73,000.

FIG. 3.5 An example of a cross-bridge between a microtubule and a vesicle (large arrow) in a pre-prophase band of microtubules. Cross-bridges between microtubules and the plasma membrane are also present (small arrows). X110,000.

FIGS. 3.6-3.9 Four serial sections through a highly stacked pre-prophase band of microtubules. Extensive cross-bridging between adjacent, transversely-sectioned microtubules is evident. X80,000.
Eleven serial sections (a-k) through a pre-prophase band of microtubules. 18 of the 42 microtubules in this part of the array terminate within these sections. Examples of microtubule terminations can be seen by following the selected numbered microtubules: microtubules numbered 1 and 3 make their last appearances in sections i and h respectively; microtubule 5 makes its first appearance in section d. A C-shaped termination is illustrated by microtubule 2 in section a. A microtubule containing a zone in which the staining is much fainter than in adjacent regions is arrowed in section d. The entire length of microtubule 4 is encompassed within these 11 sections. Microtubules passing at a greater angle than the majority in the array, e.g. those in section c (arrow heads), were not included in the mapping or calculations. Cross-bridges between adjacent microtubules and microtubules and the plasma membrane are indicated by the small arrows. X110,000.
Figs. 3.11-3.14 Microtubules and xylem development.

Fig. 3.11 Before thickening formation begins in young xylem cells, microtubules form groups along the longitudinal walls (brackets) and secondary wall deposition occurs beneath the groups of microtubules. The occurrence of different stages of development along opposite walls is explained in Section 4.3.3. Electron-dense amorphous material surrounds the microtubules over the well-developed thickenings. X25,000.

Figs. 3.12-3.14 In one sequence of serial sections, the reconstruction of which is shown in Fig. 3.13, the microtubules were grouped over two clearly discernible wall thickenings at the beginnings of the sequence (Fig. 3.12). However, by section 8 the thickenings were no longer visible. The reconstruction shows that in this region the microtubules begin to fan out, becoming more evenly distributed over the plasma membrane. Fig. 3.14 is section 11. In this map, as in all others except Figs. 3.23 and 3.25, the arrays are viewed as from the cell wall looking into the cell through the plasma membrane. In all maps, each division along the horizontal axes represents one section, estimated to be, on average, 70nm thick. Figs. 3.12, 3.14, X70,000.
**FIGS. 3.15-3.18** Microtubules in pre-prophase bands.

**FIG. 3.15** A reconstruction of part of a pre-prophase band of microtubules. The dimension normal to the cell surface is not apparent, but only 57-72% of the microtubules were adjacent to the plasma membrane. The majority of tubules lie parallel to the plasma membrane, and are not interwoven.

**FIG. 3.16** A grazing section of a pre-prophase band near a cell corner. The microtubules sectioned longitudinally can be seen to possess similar alignments to those in the reconstruction (Fig. 3.15). Vesicles are evident amongst the microtubules near the left-hand wall. X44,000.

**FIG. 3.17** Coated vesicles (arrows) were also seen amongst the microtubules in pre-prophase bands. X55,000.

**FIG. 3.18** A pre-prophase array in a root fixed in glutaraldehyde dissolved in complete polymerisation medium. Due to the complexity of this array, the mapping process has in this case involved spacing the microtubules along the plasma membrane and also straightening out their undulations.
FIGS. 3.19-3.22  Microtubules in interphase arrays (1).

The dispositions of microtubules in the reconstructions can be compared with those seen in the micrographs showing cortical microtubules adjacent to the longitudinal cell walls. In these, as in all other maps and accompanying micrographs, the axis of the root is vertically up and down the page. Terminations are generally randomly distributed throughout the arrays, however, a cluster of terminations is evident in sections 24-26 in Fig. 3.20. Fig. 3.19, X15,000; Fig. 3.21, X30,000.
Figs. 3.23, 3.24 Microtubules in interphase arrays (2).

Fig. 3.23 Scale representations of two arrays of cortical microtubules against the longitudinal cell walls at interphase. The view in Fig. 3.23A is as from the centre of the cell, looking outwards. Fig. 3.23B represents the cortex of the neighbouring cell, viewed by looking on through Fig. 3.23A and the intervening wall. The two arrays can be superimposed in register by using the left-hand frame and the asterisks as reference marks.

Fig. 3.24 A grazing section of a longitudinal wall showing similar alignment of cortical microtubules to that in Fig. 3.23. X42,000.
Fig. 3.25 A second example of two interphase arrays of microtubules in adjacent cells. Again the view in Fig. 3.25A is as from the centre of the cell looking outwards, Fig. 3.25B representing the cortex of the neighbouring cell, viewed by looking on through Fig. 3.25A and the intervening wall. The array possesses a lower density of microtubules than that in the reconstructions in Fig. 2.23.

Fig. 3.26 Transverse profiles of microtubules in interphase arrays are often cross-bridged to the plasma membrane (arrows). The section is part of the sequence mapped in Fig. 3.25. X75,000.

Fig. 3.27 Coated vesicles and polyhedral bodies (arrows) are sometimes seen amongst the cortical microtubules adjacent to the cell walls. X45,000.
FIG. 3.28  An interphase array of microtubules in a root fixed in glutaraldehyde dissolved in complete polymerisation medium (see Section 3.3.5).

FIGS. 3.29-3.31  Microtubules and low temperature treatment.

FIG. 3.29  A pre-prophase band of microtubules in a root fixed after 15 minutes at 0°C. This treatment has resulted in an overall decrease in microtubule length.

FIG. 3.30  An interphase array of microtubules in a root fixed after 4 hours at 1.6°C. Again, the average microtubule length is approximately half that in controls.

FIG. 3.31  An interphase array of microtubules in a root fixed after 15 minutes recovery following a 15 minute treatment at 2°C. The average microtubule length is in the range seen in control roots.
Fig. 3.28

Fig. 3.29

Fig. 3.30

Fig. 3.31

Number of section along sequence
FIGS. 3.32, 3.33  Microtubules and high pressure treatment.

FIG. 3.32  A pre-prophase band of microtubules in a root subjected to a pressure of 14,000 psi for 15 minutes. The array contains a large number of very short microtubules, 34% being less than 0.7µm in length, approximately three times the frequency seen in sequences of comparable length in untreated roots.

FIG. 3.33  An interphase array of microtubules in a root subjected to a pressure of 16,000 psi for 15 minutes. Roots were fixed within 30 seconds after the onset of decompression. Again, the number of very short microtubules in the array is greater than in controls.
Fig. 3.32

Fig. 3.33

Number of section along sequence
Figs. 3.34-3.36  Microtubules and colchicine treatment.

Figs. 3.34, 3.35  Interphase arrays of microtubules in roots fixed after a 2 hour (Fig. 3.34) and a 5 hour (Fig. 3.35) treatment in a $5 \times 10^{-3}$M colchicine solution. The average microtubule length was 0.8µm in both cases.

Fig. 3.36  A cortical array of microtubules in a root fixed after a 3 hour treatment in a $5 \times 10^{-3}$M colchicine solution. The array is likely to be a pre-prophase band of microtubules.
Fig. 3.34

Fig. 3.35

Fig. 3.36

FIG. 3.37  An interphase array of microtubules in a root which had been immersed in D$_2$O for 5 hours. Dispersed throughout the array is a large number of short microtubules, the presence of which lowers the calculated average microtubule length.

FIG. 3.38  An array overlying two secondary wall thickenings in a developing xylem element in material that had been treated with D$_2$O for 5 hours. There may be interpolation of microtubules amongst those over the thickenings and also between the thickenings as a result of the treatment.

FIG. 3.39  An interphase array of microtubules in a root fixed after an 18-hour treatment in D$_2$O. The average microtubule length was as in controls.
Fig. 3.37

Fig. 3.38

Number of section along sequence

Fig. 3.39
FIG. 3.40 The fraction of microtubules in an array which pass right through the sequence of sections, plotted against the number of sections in the sequence. Squares - interphase arrays; circles - pre-prophase bands; triangles - arrays overlying xylem thickenings. Open symbols - conventional fixation; closed symbols - fixation in polymerisation medium + GTP; half-closed squares - fixation in polymerisation medium without GTP. The curve was fitted by the least squares method and represents the relationship $y = e^{-0.0265s}$, where $y$ is the ordinate and $s$ is the number of sections in a sequence.
CHAPTER 4
INTERPHASE MICROTUBULE ARRAYS DURING WALL FORMATION IN AZOLLA PINNATA ROOTS

4.1 INTRODUCTION

Since Ledbetter and Porter's initial description (1963) of cortical microtubules in elongating cells in roots of Phleum, Spirodela and Juniperus, their observation of the parallel alignment of wall microfibrils and the cortical microtubules has received confirmation in many different systems (see Section 1.4.2). The orientation of the newly-deposited microfibrils is instrumental in determining the shape of the cell and the manner in which it expands (Green, 1962). Transverse orientation of these microfibrils inhibits lateral expansion, allowing only cell elongation to occur. In addition to the roots studied by Ledbetter and Porter (1963), other systems in which elongation predominates and in which transversely oriented microtubules have been observed, include the algae, Nitella (Green, 1969), Chara (Pickett-Heaps, 1967a) and Spirogyra (Powke and Pickett-Heaps, 1969), and in higher plants, during hormonally-induced elongation in lettuce (Sawhney and Srivastava, 1972) and bean hypocotyl cells (Shibaoka, 1972, 1974), in parenchyma cells in Nymphoides petioles (Freundlich, 1974); and a confirmatory report in Phleum root tip cells
Brennan, 1970). In the first four examples, transversely oriented wall microfibrils have been observed. Microfibrils are known to be oriented transversely in xylem thickenings and in every system of differentiating xylem examined, the microtubules overlying the developing thickenings are also transversely aligned (see Section 1.3.3).

Before the description of the structure of cortical microtubule arrays in *Azolla*, *Zea* and *Impatiens* root tips (Chapter 3) little, apart from their orientation and "even" or "clustered" distribution has been reported about interphase microtubule arrays in cells during primary or secondary wall deposition. The chemical composition and texture of walls varies between cell and tissue types and changes during cell development. Are these changes reflected in the organisation of the cortical microtubule arrays? Taking, for example, the system in which these microtubules were first described, i.e. young root tip cells (Ledbetter and Porter, 1963) do all newly-formed cell initials (see Section 2.3.4) possess similar numbers of microtubules in their cortical arrays? Does the density of interphase microtubules decrease as the cells elongate? After transverse proliferative division (see Section 2.3.5) is the same density of microtubules that existed in the parent cell, reinstated in the two daughter cells? Do any trends which might occur in the zone of proliferative divisions also occur during differentiation?

These are only a few questions which can be asked about cortical microtubule arrays in cells in interphase and about which there is little, if any, information. The
Azolla pinnata root provides a system in which answers to these questions can be obtained. The detailed knowledge of the root construction (Chapter 2) enables different cell types to be followed from the site of formation of the cell initial, basipetally along individual cell files, through zones of proliferation and differentiation. An analysis of the microtubule arrays in cells of different types and at different stages of development is presented in this Chapter.

4.2 MATERIALS AND METHODS

The culture of plant material and the processing of roots for light and transmission electron microscopy are as outlined in Section 2.2. Microtubule counts were made in sections of longitudinally oriented roots on parlodion coated grids with 2mm slots. Electron micrographs were taken at 10,000 - 15,000X and printed to a final magnification of 25,000 - 40,000X. Transverse microtubule profiles adjacent to the longitudinal cell walls were counted.

A number of different procedures involving the shadowing of ultra-thin sections was tried in attempts to reveal the microfibril orientation in the cell walls of the Azolla roots. One method was simply to shadow the sections after subjecting them to a vacuum of approximately 10^{-5} mm Hg for 10-30 minutes in the chamber of a vacuum coating unit (Maser et al., 1967). Sections on other grids were etched for 0.5-1 minute in sodium methoxide, rinsed in 50% ethanol and then in distilled water before shadowing. Other etched and non-etched sections were also placed in a solution of 5% hemicellulase and 5%
pectinase for 1-4 hours before shadowing. Some of these sections were stained in uranyl acetate and lead citrate prior to shadowing and some after shadowing. The sections were shadowed with platinum/carbon through a 1mm aperture at angles varying from 10° to 45°.

Scanning electron microscopy was performed on resin-embedded roots which had been trimmed using a glass knife until a median, longitudinal section was obtained. The blocks were then etched in sodium methoxide for 0.5 - 2 hours, rinsed in ethanol and coated with gold-paladium. An Hitachi HHS 2R scanning electron microscope and a JEOL 100B electron microscope in the scanning transmission mode were used to view the etched block faces.

4.3 Results

4.3.1 Microfibril Orientation

The microfibril orientation in the thin primary walls of *Azolla pinnata* cells is not readily apparent in conventionally prepared ultra-thin sections. A transverse alignment of wall microfibrils is, however, clearly evident in grazing sections of the thickened wall of the outer sieve element (Fig. 4.1). Shadowing of ultra-thin sections has been used to show the orientation of wall microfibrils in a number of plant tissues and although these usually consisted of cells with extremely thick walls, attempts were made to see
if this approach would highlight the wall microfibrils in the root tip cells of *Azolla*. A number of different procedures was tried (described in the preceding Materials and Methods (Section 4.2). The first result emerging was the finding that Spurr's resin sections did not sublume in the vacuum as did Araldite-Epon resin sections as used by Maser *et al.* (1967). Neither the Araldite-Epon sections nor etched and/or enzyme digested sections gave any good indications of microfibril orientations in the root. However, it was found that the staining, after shadowing, of enzyme-digested sections (with or without etching) did give enhanced visualisation of microfibrils over that normally seen in conventionally prepared ultra-thin sections (Figs. 4.2-4.4). These results could not be obtained if the staining was carried out prior to shadowing. Just how fortuitous or repeatable this procedure is, is not known and will require further investigation. However, parallelism and transverse alignment of wall microfibrils in the thin primary walls and of the cortical microtubules is clearly indicated. The impression of transverse alignment in the xylem secondary thickenings is evident in Fig. 4.5 and has been established using polarised light microscopy in many systems.

4.3.2 Microtubule Arrays in Cell Initials and in Zones of Cell Proliferation

The numbers of microtubule profiles in any
one section in an interphase array of microtubules was found, in most sequences of serial sections, to be relatively constant through the array (Figs. 3.20, 3.22, 3.25). On this basis, counts of microtubule profiles along longitudinal cell walls should give reasonable representations of the whole microtubule array in the cell. A possible source of error in such an analysis, however, is that immature arrays will presumably differ from mature ones. Cells examined at the final stages of cell plate formation have very few cortical microtubules along the longitudinal walls and a finite amount of time must elapse before the cortical arrays are set up. The initial analysis described here was concerned with the patterns of microtubule distributions along cell files usually commencing at the initial cell and continuing through the early zones of proliferative divisions. It is possible, therefore, that the analysis may include cells which are in the process of forming their interphase arrays and in which the microtubule counts, although they may be representative of the array as a whole, may be lower than the value for the fully-formed array. The length of time taken to set up the new arrays, however, appears to be relatively short and cells in the very early stages of this process are not often encountered.

Chapter 2 includes evidence which indicates that the sites of proliferative divisions in at least
some cell types are geared to cell elongation. The merophyte length was found to be a good measure of the stage of development of the endodermis and outer cortex, two cell types analysed in detail (Fig. 2.54). These cell types were selected because they are always represented in any median or near median longitudinal section through the root and for the same reason, the endodermis was chosen for the initial analysis, described below, of patterns of microtubule distribution during development.

Endodermis:
The endodermis forms a hexagonal ring of 6 cells around the stele (e.g. Fig. 2.14). Its outer tangential wall is flanked by cells of the inner cortex and the inner tangential wall lies adjacent to either a pericycle cell or an outer sieve element. The longitudinal sections used in this analysis cut through the inner and outer tangential walls of two files of endodermal cells, one on either side of the root (Figs. 2.13, 2.18). Microtubule profiles were counted along the longitudinal tangential walls in both files of cells, up to approximately 0.2mm from the root apex. This encompassed up to 8 merophytes. Counts were made in 5 roots, of lengths ranging from 0.63mm to 0.27mm, and for each root counts were made in 2-3 sections. Data on the merophyte length, the number of cells per merophyte and the total number of microtubule profiles along the inner and outer
tangential walls in each merophyte, are given in Table 4.1.

These data have been used to construct graphs showing the number of microtubule profiles in each cell, expressed per unit length of plasma membrane along the adjacent wall (Fig. 4.7). The length of the merophyte in which the cell occurs is used as a measure of the stage of development of the cell. Solid and open symbols in each graph represent the values along the outer and the inner tangential walls respectively. In Figs. 4.7D and E, the first value, i.e. that on the left hand side of the graph, refers to the inner tangential wall of the endodermis-inner cortex precursor cell. The first pairs of values (Figs. 4.7A-E) occur in the endodermis initial cell.

Mathematical attempts to draw curves of best fit through the points were not successful due to the apparent change of form of the curves in each case. A straight-line relationship fitted values in older merophytes in many cases but did not accommodate values in the shorter merophytes. Use of a computer to fit a simple exponential relationship was not consistently successful either, although the predicted curves did fit reasonably well in some cases. Bearing in mind the inherent errors, outlined above, further searching for a more appropriate mathematical relationship was not thought to be warranted, at least at this stage.

Three points are, however, apparent from the data
in Table 4.1 and Fig. 4.7:

(i) The general pattern of microtubule distribution along the endodermal cell files is the same in the roots of different lengths when the merophyte length is used to measure the developmental stage of the cells.

(ii) Although the number of microtubule profiles per merophyte continually increases from that in the younger merophytes (Table 4.1), there is an initial decrease in the density of microtubules in the arrays in the first 4-5 merophytes. After this initial decrease, the increase in the numbers of microtubules "keeps pace" with the root elongation to maintain an approximately constant density of microtubules along the longitudinal cell walls (Fig. 4.7). The curves plateau in the older merophytes at values of approximately 5 and 4 microtubules \( \mu m^{-1} \) for the outer and inner tangential walls respectively.

(iii) The number of microtubule profiles and thus the density of microtubules in the arrays along the inner tangential wall, is consistently lower than that along the outer tangential wall.

The 6 pairs of small symbols in the graphs refer to values of microtubule profiles \( \mu m^{-1} \) for cells not at interphase, but at pre-prophase, which had the majority of their cortical microtubules grouped in a pre-prophase band.
Inner Cortex:

The next step in the analysis of the inter-phase arrays in the *Azolla pinnata* roots was to determine if the trends in microtubule distribution that had emerged in the endodermal cell files also existed in files of other cell types. Values for the inner tangential wall of the inner cortex were available on the same micrographs as used for the endodermal counts and thus this cell type was the next examined. The results are shown in Table 4.2 and Fig. 4.8. The numbers of microtubule profiles along the outer tangential walls in the 0.37mm long root were also counted (Table 4.2) and the numbers of profiles per unit length of plasma membrane are represented by the solid symbols in Fig. 4.8D. There appears to be no significant difference between the values along the two walls and a single line has been drawn.

Again, the number of microtubule profiles increases in older merophytes (Table 4.2). Apart from the absence of a difference between the inner and outer walls, the curves showing the number of microtubules µm⁻¹ (Fig. 4.8) exhibit the same trends as did the curves for the endodermal cell files: an initially rapid decrease in microtubule density followed by a plateauing of this value, at approximately 2.5 microtubules µm⁻¹. Thus, although the pattern of microtubule distribution along the files of inner cortex cells is essentially the same as that
in the endodermis, the "base level" maintained is lower in the inner cortex than in the endodermis.

Other Cell Types:

Just as in the endodermis, the trends in microtubule distribution along the tangential, longitudinal walls of the inner cortex did not differ in roots of different lengths. It is thus likely that analyses within a single root will give reliable representations of microtubule patterns in other cell types. Five other cell files were examined in the root 0.37mm in length and the results obtained are shown in Table 4.3 and Fig. 4.9. Solid and open symbols in Figs. 4.9A and B represent values at the inner and outer tangential walls respectively, in the outer cortex and the large pericycle cell files. The values in the small pericycle and the phloem parenchyma cells represent the outer tangential walls of each file. The dermatogen wall that was analysed is that adjacent to the outer cortex. The trends in the outer cortex (Fig. 4.9A), in the small pericycle (Fig. 4.9C), and possibly in the large pericycle (Fig. 4.9B), show the rapid decrease and basal level plateauing seen in the endodermis and inner cortex. There does not appear to be any difference between distributions along the inner and outer tangential walls in either the outer cortex or the large pericycle cell files. The number of observations in the phloem parenchyma cell file
are too few to make any comment but do indicate that this cell type has approximately the same microtubule densities as the above-mentioned cells.

In the dermatogen, there is also an initial decrease, in the number of microtubule profiles $\mu m^{-1}$, but after the second proliferative division (occurring at a merophyte length of approximately $15 \mu m$), there is a rapid increase. In contrast to the situation in the other cell types so far considered, the second proliferative division in the dermatogen is followed by another formative division, the asymmetrical division which yields a trichoblast and an epidermis initial cell (see Chapter 2).

4.3.3 Microtubules and Cell Differentiation

**Outer Sieve Element:**

Both the two files of outer sieve elements and the two files of xylem elements commence, and may complete, differentiation in the zone of the root examined in the preceding section, i.e. in the zone in which the other cell types are undergoing rounds of proliferative divisions. The metaxylem initial cells do not undergo any proliferative divisions (Fig. 2.46) and are the first to enter the phase of differentiation. The outer sieve element initial cell divides once before differentiation begins (Fig. 2.46). Just as xylem differentiation proceeds in an acropetal direction towards the root apex (Fig. 4.16) so too does the differentiation of the
outer sieve elements. This means that the merophyte length will not reflect the stage of development of the cells in roots of different lengths. This is evident on comparing the microtubule distributions in the two roots in Fig. 4.15.

The number of microtubule profiles along the longitudinal walls increases after the second proliferative division (which was completed before the stages of development given in Fig. 4.15), reaching a maximum of about 400 microtubules per merophyte in the 0.37mm root (Fig. 4.15A) and about 300 microtubules per merophyte in the 0.97mm root (Fig. 4.15B). The ultrastructure of the sieve elements at the stage of development showing the maximum numbers of microtubules is illustrated in Fig. 4.10. After this, the number of microtubules in the cell rapidly decreases as the cell cytoplasm begins to break down (Figs. 4.12, 4.26, 4.27). The structure of the microtubule arrays and the transverse alignment of the microtubules are shown in the microtubule map (Fig. 4.13) and in the electron micrographs (Figs. 4.11, 4.14). The number of microtubules $\mu m^{-1}$ of plasma membrane increases after the proliferative division, reaches a maximum of approximately 12 microtubules $\mu m^{-1}$ and then rapidly declines (Fig. 4.15). There does not appear to be any difference in the microtubule distribution along the inner and outer tangential walls.
Xylem Development:

Before analysing the microtubule distributions along the files of xylem elements, it is necessary to describe in some detail the complexities of xylem development. The two files of metaxylem elements lie opposite each other in the centre of the stele (Figs. 2.14-2.17, 4.25-4.27). Initially each cell is triangular in shape (Fig. 4.26) but after hydrolysis of the common wall, the radial walls become less steeply angled and each element becomes more trapezoid in cross-section (Fig. 4.27).

As mentioned previously, the xylem initial does not divide before commencing differentiation, the course of which can be seen in Fig. 4.25 and diagrammatically in Fig. 4.40D.

After the formation of the xylem initial cell two vacuoles form at either end of the cell (Figs. 4.28, 4.40D, Stage 1). One or both of these may develop a complex system of channels giving the vacuole a lysosome-like appearance (Figs. 4.28, 4.29). Reconstructions from serial sections through these vacuoles showed that they are composed of a complex series of membrane-bound channels and chambers (Figs. 4.30, 4.31). These vacuoles remain in the cells until autolysis begins at which time they become more diffuse and break down (Stage 6, Fig. 4.40D). With the commencement of secondary wall deposition, profiles of endoplasmic reticulum (ER) are seen around the thickenings and lying parallel to the end
walls. Reconstruction from serial sections revealed that the ER between and over the thickenings may be tubular or may form 1-4 sheets (Figs. 4.35-4.37). At the end walls, 3-5 parallel sheets of ER stack against the cross-walls, particularly at the basiscopic end of the cell (Figs. 4.35, 4.36). As development continues the ER remains close to the developing thickenings but the parallel sheets at the end walls disappear. As thickening development reaches completion and autolysis begins, the profiles of ER become dilated and contain granular material which appears more electron-dense than the surrounding cytosol, this change in contrast being enhanced by the progressive autolysis of the cytosol (Fig. 4.39). During the later stages of thickening formation, there is elaboration of wall ingrowths, firstly at the end walls (Figs. 4.27, 4.38, 4.39) and then also at some of the thickenings (Figs. 2.17, 4.33, 4.34, 4.38, 4.40D, Stage 7). Both the thickenings and the wall ingrowths become lignified (Figs. 4.25-4.27).

The xylem secondary wall thickenings are in the form of discrete rings the shape of which can be seen in cleared preparations and in embedded roots which have been sectioned, etched and viewed in a scanning electron microscope (Figs. 4.33, 4.34). The outer surface of the ring conforms to the triangular shape of the young xylem elements but greater deposition of secondary wall material at the corners of the cell yields a circular central surface in cross-section
(Figs. 2.14, 2.17, 4.26, 4.27). The sites of secondary wall deposition are evenly distributed along the longitudinal wall (Figs. 3.11, 4.19-4.23, 4.37). The distance between adjacent rings increases as cell elongation proceeds (Fig. 4.24), and thus the spacing between the rings increases in cells further from the apex because of the greater elongation.

**Xylem Secondary Wall Thickening**

Secondary wall deposition begins at the corners of the xylem elements and progresses laterally around the cell (Figs. 2.12, 4.26, 4.28). The fact that in longitudinal ultra-thin sections of young elements, profiles of thickenings are often seen only on one side of the cell (e.g. Fig. 3.11) is due to this asymmetry of initial deposition. It also follows that thickening formation may have commenced at the corners of a cell even though a section passing through a face of the cell shows no signs of deposition. This situation was well-illustrated in the sequence of serial sections described in Chapter 3, the reconstruction and two sections of which appear in Figs. 3.12-3.14. In mature regions of the root where there has been a great deal of elongation, often only fragments of each ring remain. It is not known whether this results from incomplete fusion of the advancing edges of the thickening along the faces of the cell or if the rings are broken during elongation. Rings in the two adjacent xylem elements are not
necessarily "back-to-back" (Figs. 4.19-4.25, 4.38).

Xylem differentiation progresses in an acropetal direction along the roots, i.e. in longer roots, the first xylem element with secondary wall thickenings is closer to the root apex than in shorter roots (Figs. 4.16, 4.19-4.24). It is evident from the graph in Fig. 4.16 that the rate of differentiation is not constant in roots of different lengths. During the period of root growth from approximately 0.25mm to 0.35mm in length, 5-6 xylem elements in each file differentiate, whereas only 1-2 elements in each file begin secondary wall deposition as the root grows from 0.35mm to 0.45mm. However, because of the different rates of root growth (Fig. 2.50, 2.51), the differences in the above examples are not as great temporally as they might appear spatially. The former growth period takes approximately 24 hours and the latter approximately 12 hours. This means that a xylem element along each file commences thickening deposition every 4-5 hours in roots 0.25mm-0.35mm in length, and every 6-12 hours in roots 0.35-0.45mm in length. This developmental pattern culminates in different gradations of differentiation in roots of different lengths. For example, in a root 0.3mm in length, the first cell undergoing thickening formation will be 5-6 cells closer to the apex than the first cell showing stages of autolysis, with the intervening elements at intermediate stages of development. On the other hand, in a root of length
0.6mm the most acropetal cell to possess wall thickenings may immediately precede one which has begun autolysis.

This acropetal advance means that, unlike the sites of proliferative divisions, xylem differentiation (like that of the outer sieve element) commences in cells of different lengths, i.e. at different merophyte lengths (Figs. 4.17, 4.19-4.24). Thickening deposition commences in progressively shorter cells until the root is about 1.4mm in length. Fewer thickenings are initiated in the shorter cells (Figs. 4.19-4.24) and a linear relationship was found to exist between the number of thickenings and the cell length at commencement of secondary wall deposition (Fig. 4.18). This results in a constant spacing between the thickenings at early stages of development, approximately 2 µm between adjacent bands. Differential elongation along the root (Fig. 2.42) subsequently generates a gradation of separation of the rings along the root, the separation increasing towards the base of the root.

The microtubule patterns in differentiating xylem elements (as in the outer sieve elements) can thus not be directly compared between roots of different lengths (Fig. 4.40A,B,C). The pattern is more expanded, and is shifted to the right, i.e. to longer merophyte lengths, the shorter the root. After the formation of the xylem initials, the number of microtubules per µm along the longitudinal
walls initially decreases, as was the case in the cell types examined in the preceding section (4.3.2). However, the decrease is followed by an increase in the number of microtubules \( \mu m^{-1} \), preceding the commencement of secondary wall deposition (arrows). The maximum microtubule density (about 7-8 microtubules \( \mu m^{-1} \)) occurs when the wall thickening deposition is approximately one half to two thirds complete, and after this, the number and density of microtubules in the cells rapidly decreases (Fig. 4.40D).

In the xylem initials and sometimes the next 1 or 2 cells (dependent, as explained above, on the root length) the cortical microtubules are distributed relatively evenly along the longitudinal walls. In older cells, but before (in time and/or space) any thickening formation is evident, the microtubules become grouped into a varying number of bands (Fig. 3.11). The exact number of bands is, as explained above for the number of thickenings, dependent on the cell length at the time of initial deposition of the secondary wall bands. As secondary wall deposition proceeds the microtubules remain grouped over the thickenings (Figs. 3.11, 3.12, 4.32, 4.37) and are often surrounded by electron-dense, amorphous material (Fig. 3.11). At early stages of development the wall bands possess an electron-translucent space between the stained wall material and the plasma membrane (Figs. 3.11, 4.37, Stages 4 and 5, Fig. 4.40D). As development proceeds the entire thickening becomes
stained (Stages 6 and 7, Fig. 4.40D). The wall ingrowths also show this same pattern of development (Fig. 4.34). As wall deposition ceases and autolysis begins the number of microtubules overlying the thickenings decreases (Figs. 4.39, 4.40).

The ultrastructural changes that occur during differentiation allow a series of arbitrary stages of development to be assigned to the cells in the files seen in each category of root length. These arbitrary stages in differentiation are diagrammed below the graph in Fig. 4.40D, which relates them to trends in microtubule distribution.

4.4 DISCUSSION

Until now, studies related to microtubules and wall formation have been largely preoccupied with descriptions of whether or not microtubules are present in the cortical cytoplasm adjacent to developing cell walls, and with their orientation: whether or not the microtubule alignment parallels the alignment of the most recently deposited microfibrils in the wall. In the majority of cases cortical microtubules are not only present, but are parallel to the wall microfibrils. Supposed "exceptions" to this generalisation have sometimes been emphasised (O'Brien, 1972; Hepler and Palevitz, 1974), but the impression of exceptional behaviour is in large part due to the general tendency to imagine the cortical microtubules as static, continuous hoops or spirals. Cortical microtubules are not hoops (Chapter 3) and it is unlikely that the microtubule arrays
are static. On the contrary, quite apart from the regular cycle of appearance following cytokinesis and their disappearance during mitosis, with the intermediacy of the pre-prophase band, the arrays must be dynamic not only in terms of a constant turnover of microtubule subunits in the equilibrium assembly-disassembly of the tubules themselves, but also in the changes of organisation of the array as a whole. This latter aspect of the dynamic nature of the microtubule arrays is well-illustrated in *Oocystis* where changes in orientation of the microfibrils between alternate lamellae are correlated with similar changes in microtubule orientation (Robinson *et al.*, 1976), or in the changes of microtubule alignment induced by applied hormones (e.g. Shibaoka, 1972; Sawhney and Srivastava, 1974). Such changes in microtubule orientation are relatively obvious, but quantitative data on microtubule abundance as related to growth and increased or decreased cellulose deposition are less easily obtained and have received very little attention.

4.4.1 Microtubules and Primary Wall Formation

During cell plate formation, phragmoplast microtubules are oriented perpendicular to the plane of the developing cell plate which is formed by the fusion of vesicles and which contains very little cellulose (Roelofsen, 1959). Following fusion with the parental cell walls deposition of cellulose in the cell plate increases its cellulose content to that characteristic of the particular cell or tissue type (qualified
below). In *Sphagnum* leaflets a "post-cytokinetic" layer of microtubules was observed (Schnepf, 1972, 1973, 1974): immediately following cell division, the distribution of cortical microtubules was uneven around the cell profile, the majority of microtubules lying adjacent to the new cell wall. Later stages of development showed more even distributions. This clearly also occurs following the formative divisions that generate the longitudinal walls studied in the present work (Figs. 4.7-4.9, 4.40A,B,C). The number of microtubules per µm against the newly-formed wall is greater than the number that occur later in the development of the same wall, i.e. in the zone of transverse, proliferative division of each cell type, before cell differentiation commences. The logic behind the proposed cytoskeletal role for the "post-cytokinetic" layer of microtubules in *Sphagnum*, which is supposed to "stiffen the young wall until it has attained sufficient stability" (Schnepf, 1974), is arguable. It seems equally appropriate that the greater initial number of microtubules could be correlated with the initiation of cellulose deposition and orientation that must be occurring at the new cell plate.

The longitudinal walls in the young *Azolla* root tip cells are elongating rapidly and provide an excellent system in which to seek evidence of any trends in microtubule distribution during primary wall formation before cell-specific wall
differentiation occurs. In the zone of proliferative divisions for the cell types examined, i.e. where wall deposition is "keeping pace" with cell elongation, the number of microtubules adjacent to the wall decreases subsequent to the initial high value seen soon after cell plate formation, and then plateaus, the value of the base level apparently being dependent not only on the cell type but also on the position of the wall of the cell. This latter qualification is exemplified by the endodermal cells. In these cells, there are greater numbers of microtubules in the arrays adjacent to the inner cortex cells than along the inner tangential walls adjacent to the stele. This situation would be impossible if the microtubules were "hoops". Although the spread of the points in the graphs is considerable, there may also be a difference in microtubule frequencies in the different cell types. The inner tangential wall of the endodermis is the outer wall of the large and small pericycle cells. A value of approximately 3.5 microtubules µm⁻¹ in the endodermis compares with a value of approximately 3.0 microtubules µm⁻¹ in the pericycle cells. Along the outer tangential wall of the endodermis a value of approximately 4.5 microtubules µm⁻¹ in the endodermis compares with approximately 2.5 µm⁻¹ along the inner tangential wall of the inner cortex. In contrast with the difference along the two opposite walls in the endodermis, both inner and outer tangential
walls in the inner and outer cortex and in the large pericycle possess approximately equal numbers of microtubules per µm of plasma membrane. The differential microtubule distributions observed may correlate with different amounts of cellulose being deposited in the cell walls. Strong transverse reinforcement of the endodermal walls may be involved in the very restricted lateral expansion of the stele, most of the increase in girth of the root occurring in the inner and outer cortical layers.

A very different development of the cortical microtubule arrays occurs when the outer sieve element begins differentiation and starts to deposit a thick cell wall. With the increase in wall deposition there is an increase in the frequency of microtubules along the wall, reaching a maximum of more than twice that in the other cell types before the cell cytoplasm begins to break down. Thus, in cells of Azolla root tips that are engaged in primary wall formation, cortical microtubules not only reflect the microfibril orientation but their numbers also correlate with the amount of cellulose being deposited. This does not mean that the microtubules are required for cellulose deposition. Greater deposition of cellulose may require greater numbers of microtubules for orientation or conversely, when less cellulose is being deposited, fewer microtubules may be needed to govern orientation. Ratios of the number of cortical
microtubules to the number of recently-deposited wall microfibrils have been calculated in the algae, *Poteriochromonas* (Schnepf *et al*., 1975) and *Oocystis* (Robinson *et al*., 1976; Sachs *et al*., 1976). A 1:1 relationship between microtubules and microfibrils of chitin was observed in *Poteriochromonas*. In *Oocystis*, a ratio of microtubules to cellulose microfibrils of 1:2.5 was estimated. In *Azolla*, if the cortical microtubules were simply serving a cytoskeletal function, as suggested in *Sphagnum* by Schnepf (1973, 1974), it is difficult to imagine why their distribution along cells which are undergoing the same amount of extension, would not be the same no matter what the cell type nor how much wall material was being deposited.

4.4.2 Microtubules and Secondary Wall Formation

Concentrated arrays of microtubules form adjacent to the cell plate that becomes the longitudinal wall of the new xylem elements. The number of microtubules per µm of plasma membrane then decreases as the cell undergoes a period of elongation and primary wall formation. Then, prior to secondary wall deposition, the number of microtubules increases at a greater rate than that at which the plasma membrane is increasing and the number of microtubules µm⁻¹ increases markedly. Many, but not all, of the microtubules group into bands along the walls, the number of groups and subsequent number of thickenings
being dependent on the cell length at this time.
Thickening formation then proceeds beneath the bands of microtubules just as described in many other systems that have been studied (see Section 1.4.4). During all stages of thickening formation there are a few microtubules adjacent to the elongating primary wall between the thickenings. Thus, in *Azolla*, orientation of cellulose in the primary wall between the developing xylem thickenings may also occur, and be under the influence of cortical microtubules.

In *Azolla pinnata*, it has been possible to determine the exact sites of initiation of thickening formation within the cell, i.e. not only the zones along the wall predicted by the microtubule clusters, but also the sites around the cell, namely at the three corners of the triangularly-shaped cell. Such a pattern of initiation explains why a profile of a developing xylem element, in an ultra-thin section, often possesses thickenings (or clusters of microtubule profiles) only on one side of the cell. It is likely that microtubule clustering and subsequent secondary wall deposition begin at localised sites around the cell in other systems as well. Thickening formation might, for example, begin preferentially on a wall adjacent to an already differentiating element - indeed it could be envisaged that some form of intercellular communication from the differentiating element could trigger the onset of xylem
differentiation in the recipient cell. Studies of the effects of colchicine on differentiating xylem elements in *Coleus* (Hepler and Fosket, 1971; Hepler and Palevitz, 1974) showed that if the cells were at a very early stage of differentiation at the time of colchicine treatment, a thin secondary wall was smeared over the primary wall throughout the cell. On the other hand, if the microtubules were removed by the colchicine after the pattern of wall bands was established, then irregularly shaped thickenings were formed. If microtubules cluster and establish the zones for secondary wall deposition initially at localised sites along a wall adjacent to another xylem element then the apparent influence of thickenings in the adjacent cell in the absence of microtubules (Hepler and Fosket, 1971) might be explained. It would simply be that the zones for subsequent thickenings had been established by clusters of microtubules along the wall adjacent to another xylem element, but the pattern had not yet been established around the entire cell circumference before the colchicine treatment removed the microtubules.

Many mechanisms have been proposed by which microtubules bring about the orientation of cellulose microfibrils in the secondary wall bands in developing xylem elements. Generally, these proposals fall into two categories: (1) interaction of the microtubules with Golgi- or ER-derived vesicles, containing wall materials, either by (a) acting as a barrier
through which the vesicles cannot pass (Robards, 1968; Goosen-de Roo, 1973), or (b) by channelling or trapping the vesicles (Pickett-Heaps, 1968; Robards and Kidwai, 1969; Maitre and De, 1971; Srivastava and Singh, 1972; Brower and Hepler, 1976) and (ii) direct interaction with the plasma membrane or membrane-bound enzyme complexes (Cronshaw, 1967; Hepler and Fosket, 1971; Brower and Hepler, 1976). In developing xylem elements in *Azolla*, vesicles have been seen mainly at the edges of the developing thickenings. Bridges between the microtubules overlying the thickenings and the plasma membrane were also observed (Fig. 3.12). The possible function of microtubules in the orientation of cellulose microfibrils during wall formation will be discussed further in Chapter 7.
**Table 4.1** The number of microtubule profiles per merophyte along the inner and outer tangential walls in endodermal cell files.

<table>
<thead>
<tr>
<th>Merophyte length (µm)</th>
<th>Number of cells in merophyte</th>
<th>Number of microtubule profiles along the Inner Tangential Wall</th>
<th>Number of microtubule profiles along the Outer Tangential Wall</th>
</tr>
</thead>
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<td>1</td>
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<td>58</td>
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Table 4.2 The number of microtubule profiles per merophyte along the inner and outer tangential walls in inner cortex cell files.

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<tr>
<th>Merophyte length (µm)</th>
<th>Number of cells in merophyte</th>
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<th>Number of microtubule profiles along the Outer Tangential Wall</th>
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Table 4.3 The number of microtubule profiles per merophyte along the inner and outer tangential walls in outer cortex and large and small pericycle cell files.

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<th>Cell Type</th>
<th>Merophyte length (µm)</th>
<th>Number of cells in merophyte</th>
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Figs. 4.1-4.6 The orientation of microfibrils in the longitudinal walls of *Azolla* root tip cells.

In all 5 micrographs, the longitudinal axis of the roots runs vertically up and down the page.

**Fig. 4.1** A grazing section of the thickened wall of a developing outer sieve element. The transverse alignment of wall microfibrils is evident. Transversely aligned microtubules can also be seen in the cortical cytoplasm adjacent to both longitudinal walls. X30,000.

**Fig. 4.2-4.5** Ultra-thin Spurr's resin sections which have been digested with hemicellulase and pectinase for 1 hour, shadowed with Pt/C and then stained with uranyl acetate and lead citrate. The sections in Figs. 4.4 and 4.5 were etched in sodium methoxide prior to enzyme digestion. Transverse alignment of wall microfibrils is clearly evident. The cells were not identified and may have been either cortical or stelar cells. Transversely aligned cortical microtubules are also visible in the non-etched preparations (arrows). Fig. 4.2, X45,000; Figs. 4.3, 4.4, X35,000; Fig. 4.5, X40,000.

**Fig. 4.6** The impression of transverse alignment of wall microfibrils in the xylem thickenings is gained from this micrograph. Cortical microtubules overlying the thickenings are also transversely oriented. X36,000.
The number of microtubule profiles per µm along the longitudinal cell walls in endodermal cells at different stages of development, as measured by the merophyte length in which the cell occurs. The graphs in Figs. 4.7A-E give data for 5 roots of different lengths: A - 0.63mm; B - 0.53mm; C - 0.44mm; D - 0.37mm; E - 0.27mm. Solid symbols represent values along the outer tangential walls (i.e. those adjacent to the inner cortex) and open symbols, values along the inner tangential walls (i.e. those adjacent to the stele).

The 6 pairs of smaller symbols represent values of microtubules per µm average over the length of the longitudinal cell walls for cells at pre-prophase, in which the majority of microtubules are grouped into pre-prophase bands. In Figs. 4.7D and E, the first open symbol on the left-hand side of the graph gives the value against the inner tangential wall of the inner cortex-endodermis precursor cell. In all graphs the first pairs of symbols represent values in the endodermal initial cells. The first and second proliferative divisions occur at merophyte lengths of approximately 20µm and 35µm respectively.
FIG. 4.8 Microtubule distributions during development in inner cortex cells.

The number of microtubule profiles per µm along the longitudinal cell walls in inner cortex cells, at different stages of development. Root lengths as in Fig. 4.7. Solid symbols: values along the outer tangential wall adjacent to the outer cortex. Open symbols: values along the inner tangential wall adjacent to the endodermis. First, second and third proliferative divisions (the latter in the most acroscopic cell of each merophyte, only) occur at merophyte lengths of approximately 10µm, 15µm and 20µm respectively.
FIG. 4.9 Microtubule distributions during development in the outer cortex, pericycle, phloem parenchyma and dermatogen layers.

All data are from the root 0.37 mm in length (see Figs. 4.7D and 4.8D). Solid and open symbols in A and B represent outer and inner tangential walls respectively. In D values occurred along the wall adjacent to the endodermis and in E, along the wall adjacent to the outer cortex. First and second proliferative divisions in the outer cortex occur at merophyte lengths of approximately 15 µm and 20 µm respectively.
FIGS. 4.10-4.14  Microtubules and development in the outer sieve element cells (1).

FIG. 4.10  A longitudinal section of an outer sieve element at the stage of development in which maximum numbers of cortical microtubules are seen (see Fig. 4.15). X8,500.

FIG. 4.11  A transverse section of an outer sieve element at a very early stage of differentiating, before any wall thickening is evident. Vesicles and densely-staining amorphous material are associated with the transversely aligned microtubules near the corners of the cell. X20,000.

FIG. 4.12  A longitudinal section of an outer sieve element at a late stage of differentiation. Autolysis of the cytosol has begun and fewer microtubules occur adjacent to the thickened walls (see Fig. 4.15). X7,600.

FIG. 4.13  A reconstruction of an array of microtubules in a differentiating outer sieve element cell. The calculated average microtubule length in this array was 5.1 µm. Two short, complete microtubules have been encompassed in the sequence of sections.

FIG. 4.14  A longitudinally-sectioned outer sieve element cell near a corner of the cell showing the transversely aligned microtubules, as in the reconstruction. X40,000.
Number of section along sequence
The number of microtubule profiles per µm along the longitudinal walls of differentiating outer sieve element cells. Root in A - 0.63mm long; B - 0.37mm long.

The outer sieve element cell initials enter one round of proliferative division before differentiation begins. This division has been completed at merophyte lengths shorter than those in which microtubule counts have been obtained (Figs. A and B). Because differentiation progresses acropetally along the root, the merophyte length is not a measure of the stage of development of the cells.
A

Outer sieve element

0.37 mm

Microtubules pm⁻¹

B

0.63 mm

Merophyte length pm
Figs. 4.16-4.24 Xylem differentiation. (1) The acropetal progression of xylem differentiation.

Fig. 4.16 The distance (measured in numbers of mero­phytes) of the most acroscopic element in the files of xylem cells, possessing signs of thickening formation, in roots at different stages of development.

Fig. 4.17 The length of the most acroscopic cell in which thickening has commenced, in roots at different stages of development.

Fig. 4.18 The number of thickenings in xylem elements at early stages of differentiation, as a function of the cell length.

Figs. 4.19-4.24 The data in Figs. 4.16-4.18 are exemplified in these cleared roots observed using Nomarski interference-contrast optics. The xylem elements at the right of Fig. 4.19 are situated at the junction of the plant and the root. The sequence of roots is from the youngest (Fig. 4.19) to the oldest (Fig. 4.24). All X500.
Figs. 4.25-4.27 Xylem differentiation. (2) Lignification of the secondary wall thickenings and wall ingrowths.

Xylem elements at different stages of development can be seen in the longitudinal (Fig. 4.25) and transverse (Figs. 4.26, 4.27) sections, stained with Toluidine Blue O. Lignified walls stain light blue and non-lignified walls stain a purple colour with this metachromatic stain. The secondary wall thickenings and wall ingrowths in the xylem are stained light blue indicating that these walls have been lignified. The wall ingrowths at the transverse walls can be seen in the longitudinal section (Fig. 4.25) and the transverse section in Fig. 4.27.

The section in Fig. 4.26 is in a younger zone of the root than that in Fig. 4.27. The outer edge of the annular thickening conforms with the triangular shape of the cell, but the inner edge is circular in cross-section. Early stages of wall thickening are also evident at the corners of an outer sieve element (Fig. 4.26). This latter wall is not lignified. The outer sieve elements have completed their differentiation in this region of the root. Fig. 4.25, X850; Figs. 4.26, 4.27, X1,100.
FIGS. 4.28-4.34  Xylem differentiation. (3) Vacuoles and the secondary wall rings.

FIGS. 4.28-4.31  Lysosome-like vacuoles often form in the young xylem cells before any wall thickening formation is evident. Longitudinal (Fig. 4.28) and transverse sections (Fig. 4.29) and reconstructions from serial longitudinal sections (Figs. 4.30, 4.31) showed that the vacuoles consist of a complex system of channels and chambers. Fig. 4.28, X7,000; Fig. 4.29, X4,000; Fig. 4.30, X10,000; Fig. 4.31, X40,000.

FIG. 4.32  Microtubules overlying the developing thickenings are transversely oriented, the long axis of the root extending across the page. Coated vesicles (arrows) sometimes occur amongst the microtubules near the edges of the thickenings. X34,000.

FIG. 4.33, 4.34  Scanning electron micrographs of etched block faces after trimming the block until the centre of the root was exposed. Complete and broken rings and the wall elaborations on the thickenings (black arrows) and at the transverse wall (white arrow) are visible. Fig. 4.33, X700; Fig. 4.34, X3,500.
Figs. 4.35-4.39  Xylem differentiation. (4) Endoplasmic reticulum and wall ingrowths.

In all figures, the apex of the root lies to the right of the page.

Figs. 4.35, 4.36  Reconstructions from serial, longitudinal sections demonstrate the stacking of sheets of E.R. against the transverse walls and between and over the developing thickenings in young xylem elements. Tubular forms of E.R. also occur throughout the cells. Both, approx. X10,000.

Fig. 4.37  Extensive stacking of profiles of E.R. occur over and between the thickenings and also at a distance from the walls. X14,000.

Fig. 4.38  A cleared root observed using Nomarski interference-contrast optics. Wall elaborations occur at the transverse walls (arrows) and along the central wall between the 2 files of xylem elements. X550.

Fig. 4.39  Dilated profiles of E.R. completely envelope the developing wall ingrowths. The xylem element on the left is at a more advanced stage of development than that on the right. Microtubules can be seen near the wall ingrowths in both cells. X25,000.
Fig. 4.40 Xylem differentiation. (5) Microtubule distributions.

A,B,C: Because xylem differentiation progresses acropetally along the root the merophyte length is not a measure of the stage of differentiation of the cell. There are no proliferative divisions in the xylem initial cell before differentiation begins. The arrows indicate the length of the merophyte containing the first signs of secondary wall deposition.

D: The data on microtubule frequencies from roots of different lengths have been pooled by assigning arbitrary stages of development based on ultrastructural appearance of the cells. The stage immediately following the final formative longitudinal divisions in which there is an initially high number of microtubules (left-hand points in Figs. B,C) has not been included. The microtubule distributions can be related to the seven arbitrary stages of development diagrammed below the graph.
5.1 INTRODUCTION

Three years after microtubules had been observed in the cortex of plant cells in interphase and in the spindles of dividing cells (Ledbetter and Porter, 1963), a specialised array of microtubules was observed in certain plant cells at early prophase (Pickett-Heaps and Northcote, 1966b, c). It was found that the plane of cell division was predicted by this transitory band of microtubules, which encircles the cell where the future cell plate will become attached to the parental walls. The initial reports described this "pre-prophase band" to be about 2.6 µm in width and to contain 150 or more microtubules often stacked 3-4 microtubules deep. Many subsequent observations of pre-prophase bands have been made in a wide range of plant tissues, but their function has remained an enigma and it has not become clear whether they occur in all types of division or only in certain specialised situations. The positions of some pre-prophase bands were indicated in the diagrams of two transversely-sectioned A. pinnata roots in Chapter 2 (Fig. 2.55). The mapping of serial transversely-sectioned microtubules in many pre-prophase bands (Chapter 3) has yielded a different picture of their structure than
formerly envisaged, and may in part explain why the above-mentioned uncertainties have remained. The size of pre-prophase bands in *Azolla* can vary between 30 to >100 microtubule profiles and can change markedly even within single band (Figs. 3.15, 3.18). Thus, when searching for pre-prophase bands, it is necessary to avoid the pre-conceived idea that they contain 150 or more hoop-like microtubules.

The location and orientation of all classes of cell division in the *Azolla pinnata* root has been determined (Chapter 2) and, combined with the newly-gained knowledge of the geometry of these microtubule arrays (Chapter 3), this basic information is used here to study the distribution of the pre-prophase band and aspects of its development and function. The materials and methods used were as described in Section 2.2.

5.2 RESULTS

The *Azolla* root offers many advantages for the study of these transitory microtubule arrays which form before prophase. The pattern of already existing walls can be followed towards the root apex in order to find precisely where the next division will occur and that site can then be examined. For most of the root, the divisions are transverse and proliferative, but close to the apex the same procedure can be applied to formative as well as to proliferative cell divisions.
5.2.1 Pre-prophase Bands in Proliferative Divisions

Already formed transverse walls in older merophytes and sometimes within the same merophyte indicate where the next transverse walls will be laid down. Both these situations are exemplified in Figs. 5.2-5.6. Fig. 5.2 shows pre-prophase bands in two dermatogen cells and in an outer cortex cell and the exact type of these divisions is indicated by reference to Fig. 5.3. All three cells are undergoing second proliferative divisions and the positions of the future cell plates are predicted by the pre-prophase bands, as shown by reference to the next older merophyte in the light microscope section.

A similar example is given for a proliferative division in the stele. Figs. 5.4-5.6 show two sections through a pre-prophage band for the second proliferative division in a pericycle cell, cutting through the band near a cell corner. The second section is two sections away from the first in a sequence of serial sections. Again, the pattern of transverse walls in the pericycle in the next older merophyte shows that the pre-prophage band predicts the position of the new wall. In this case, the position is also indicated by the pattern of transverse walls within the same merophyte, i.e. a stepping of existing transverse walls across the root from the inner cortex to the centre of the stele. The pre-prophage band lies in the expected position.

Almost certainly every proliferative division
in the root is preceded by a pre-prophase band. They have been observed prior to most (in some cases all) rounds of division in the dermatogen, epidermis, outer and inner cortex, endodermis and pericycle, and in at least some of the remaining stelar files.

5.2.2 Pre-prophase Bands in Formative Divisions

The division following the two rounds of proliferative divisions in the dermatogen is extremely asymmetrical, cutting off a small inner basiscopic corner of each dermatogen cell and forming a trichoblast and an epidermal initial cell (Fig. 5.3). The pre-prophase band (Fig. 5.1) accurately anticipates the asymmetry of this division, which proceeds acropetally along the files of pre-existing dermatogen cells at a rate of one completed division approximately every 100 minutes. This cell-by-cell progression yields an ideal situation for the study of the development of a pre-prophase band. Fig. 5.7A shows the microtubule distribution along the central portion of the transverse walls in a file of dermatogen cells, from the cell immediately adjacent to the first completed divisions (left-hand side) to a cell eleven cells closer to the apex (right-hand side). The orientation is the same as that in Fig. 5.3 although there are fewer merophytes which have completed the second proliferative division in the root shown in the micrograph. Pre-prophase bands, in varying degrees of development, are apparent.
up to 7 or 8 cells along the cell files in advance of the last completed division. That the pre-prophase band forms as the interphase array of cortical microtubules begins to break down is suggested by the counts of the numbers of microtubule profiles on either side of these transverse walls (Fig. 5.7B). Eleven cells from the first completed division the number of microtubule profiles along both transverse walls of the cell is approximately equal. As the pre-prophase band develops, exemplified by progressing basipetally along the file of dermatogen cells, the number of microtubule profiles along the acroscopic, transverse walls decreases as that along the basiscopic wall, i.e. the transverse wall occupied by the band, increases. As shown by the distribution pattern in Fig. 5.7A, the increase in the number of microtubules, graphed in Fig. 5.7B, is due to the formation of the pre-prophase band.

Four *A. pinnata* root tips were transversely sectioned at frequent enough intervals to allow every cell in the formative zone to be examined. Of the total of 628 cells, 26 had pre-prophase bands, and only two were in post-prophase stages of mitosis. Pre-prophase bands occur in all 10 categories of formative divisions in the *A. pinnata* root meristem. Systematic surveys were made by following longitudinal walls down a sequence of merophytes until cells were reached, in the zone of formative divisions, where the divisions which give rise to those walls
had not yet been completed. The position of the future walls was predictable from the line of the existing walls. The pre-prophase bands were found to span these sites. As an example, the pre-prophase bands that preceded every division along the cell lineage that leads to the formation of the endodermal and inner cortex initial cells (Fig. 2.47) are illustrated and described below.

The Endodermal and Inner Cortex Cell Lineages

All of the cell lineages start at the apical cell. Its divisions are asymmetrical, and occur in a left-handed or right-handed sequence. The face where the next division will occur is indicated by the pattern of overlap of the already existing walls (Fig. 5.8). The pre-prophase band (Figs. 5.9, 5.10) is parallel to the face of the apical cell where the next division would have occurred. The cell plate which forms following this division (Fig. 5.11), and subsequent pre-prophase bands, are illustrated in longitudinal sections of the root.

Newly-formed merophytes have two large triangular faces (acroscopic and basiscopic), narrow radial longitudinal walls, and an outer curved face occupying 120° of the circumference of the root (Insert, Fig. 5.11). The first division, tangential-longitudinal, is accomplished by a curved cell plate (Insert, Fig. 5.12) and is preceded by a pre-prophase band as seen along the acroscopic and basiscopic
faces in Fig. 5.12.

The cell lineages now diverge and a radial-longitudinal division in the inner cell forms two sextant cells (Insert, Fig. 5.13). The pre-prophase band preceding this division (Fig. 5.13) has been cut in the plane of the band in a longitudinal section of the root. The cell in the adjacent basiscopic merophyte has completed this division and has formed the pre-prophase band for the next, tangential-longitudinal division (Insert, Fig. 5.14). The pre-prophase band along this acroscopic wall and also along the basiscopic wall of the same cell, is shown in Figs. 5.14 and 5.15 respectively. The cell in the merophyte adjacent to the basiscopic wall has completed this division and the pre-prophase band is seen to be positioned in line with the longitudinal wall in the older merophyte. The final division is also in the tangential-longitudinal plane (Insert, Fig. 5.16). Again, the position of the pre-prophase band is seen to be in line with the existing wall in the next older merophyte (Figs. 5.16-5.18). A transverse section showing the first merophyte to have completed all the divisions of the endodermal and inner cortex cell lineages is illustrated by a light microscope section in Fig. 5.19. At this level of the root, the merophytes in the other two 120° sectors have yet to complete the final tangential-longitudinal division.
5.2.3 Microtubules in Pre-prophase Bands

The detailed reconstructions derived from serial sections described in Chapter 3 have shown that pre-prophase bands in *Azolla* roots consist of short, overlapping, cross-bridged microtubules. The fact that the constituent microtubules are not long enough to encircle the cells can explain the disparity in microtubule numbers that is frequently encountered when a pre-prophase band is examined on opposite sides of the same cell. Despite the common occurrence of these asymmetrical bands, symmetrical bands were also found in every category of division examined in detail. A number of sequences of serial sections described in Chapter 3 encompassed quite rapid changes in the number of microtubules in the pre-prophase bands (e.g. Figs. 3.15, 3.18). The observed disparities in microtubule numbers may represent stages in the formation or breakdown of a more or less symmetrical state.

It is difficult, therefore, to estimate an average for the number of microtubule profiles in a pre-prophase band. The maximum number seen was 156, but symmetrical bands with far fewer than this were common. The number of microtubules may vary from one class of division to another and may be related to cell size. Some bands show pronounced stacking of microtubules while others contain little more than a monolayer of microtubules. Nearly all well-formed bands did, however, occupy a 1.5-3 μm wide band of
the cell cortex. Small vesicles (Figs. 5.10, 5.12-5.18), and sometimes dark staining amorphous material (Figs. 5.12, 5.15), can occur amongst the pre-prophase band microtubules.

5.3 DISCUSSION

5.3.1 Introduction

Several functions of the pre-prophase band have been proposed. Burgess and Northcote (1967), and Burgess (1970c) argue that the band participates in positioning the nucleus prior to mitosis, this aligning influence perhaps persisting until the mitotic poles are well developed (Burgess, 1969). Pickett-Heaps (1974) favours the view that, while it reflects the way in which the cell has become polarised, it also represents a pool of tubulin and/or microtubules that is drawn upon during spindle formation. Palevitz and Hepler (1974a) suggest that the band itself is a by-product of the establishment of a cortical microtubule organising zone that will function later in cell division, producing microtubules which will guide the growth and fusion of the cell plate. Packard and Stack (1976) observed vesicles amongst the pre-prophase microtubules and believe that the underlying cell wall becomes locally thickened, subsequent wall expansion being more or less limited to this zone so that the new cell plate and the walls to which it fuses become H-shaped inserts into the pre-existing wall. The apparent absence of pre-prophase
bands prior to some divisions has added to the uncertainty of their function. The detailed study of pre-prophase bands in *Azolla pinnata* has revealed much information relevant to their distribution and to their possible function. As the occurrence of pre-prophase bands is in itself informative in considerations of function, the *Azolla* results will first be examined in relation to the relevant literature on the anatomical and taxonomic distribution of pre-prophase bands.

5.3.2 Occurrence of Pre-prophase Bands

The cell lineages in *Azolla* root tips are longer and more complex than any in which pre-prophase bands have previously been sought. They include symmetrical and asymmetrical divisions; divisions in longitudinal, transverse, radial, tangential, and oblique planes; and formative as well as proliferative division cycles. Pre-prophase bands are present prior to all categories of division, accurately anticipating the site of fusion of the future cell plate with the parental wall. The ubiquity of pre-prophase bands in *Azolla pinnata* raises the question: do all cell divisions require pre-prophase bands, and if not, can the distribution patterns be rationalised meaningfully? The known distribution is as follows:

*Roots:* *Azolla*, present in symmetrical and asymmetrical cell divisions (present work);

*Triticum*, unspecified divisions (Pickett-Heaps
and Northcote, 1966b; Burgess, 1969);
*Allium*, unspecified divisions (Deysson and Benbadis, 1968; Mesquita, 1970; Benbadis *et al.*, 1974; Packard and Stack, 1976); *Phleum*, unspecified divisions (Burgess and Northcote, 1967); *Dryopteris* (Burgess, 1970d).

*Leaves and cotyledons*: *Nicotiana* mesophyll (Cronshaw and Esau, 1968; Esau and Gill, 1969); *Commelina* mesophyll, epidermis, and stomatal complexes (Pickett-Heaps and Northcote, 1966c; Pickett-Heaps, 1969c); *Avena* stomatal complexes (Kaufman *et al.*, 1970); *Allium* stomatal complexes (Palevitz and Hepler, 1974a).

*Cambium*: Ray cells in *Ulmus* (Evert and Deshpande, 1970).

*Bryophytes*: *Sphagnum* leaves (Schnepf, 1973; *Marchantia*, developing mucilage papillae (Galatis and Apostolakos, 1977).

All the above "positive" reports concern two- or three-dimensional parenchymatous tissues, a preliminary generalisation which assumes more significance when the "negative" reports are considered. Hepler and Palevitz (1974) list several publications in which pre-prophase bands were sought but not found,
or where they were not reported. Some may reflect genuine absences, but others may mean no more than that the investigators did not find cells at the correct stage of division. For example, Cutter and Hung (1972) present a negative report for the asymmetrical division that gives rise to trichoblasts and atrichoblasts in *Hydrocharis morsus-ranae* roots. However, both this division and symmetrical transverse divisions in internal cell files in the closely related *H. dubia*, have been found to possess typical pre-prophase bands (Gunning, Hardham and Hughes, 1978a). It seems likely that the failure to find the band in fusiform initials in *Ulmus* cambium (Evert and Deshpande, 1970) is in a similar category.

The situations where absence would appear to be genuine include both algae and higher plants. No pre-prophase band has been reported for an alga, whether the divisions be mediated by phycoplasts or phragmoplasts. There are, however, many parenchymatous types, particularly in the brown algae, that remain inadequately or not at all investigated. *Chara* has been thoroughly examined but no band found (Pickett-Heaps, 1967a).

In higher plant material, typical pre-prophase bands have not been reported to occur during formation of vegetative and generative cells in microspores (e.g. Burgess, 1970b; Sanger and Jackson, 1971; Cass and Karas, 1975), during sperm formation in pollen (Burgess, 1970c; Cass and Karas, 1975), in preparations
of liquid endosperm (Bajer and Mole-Bajer, 1972), and during cellularisation of endosperm in situ (Newcomb and Fowke, 1973; Morrison and O'Brien, 1976). Two other negative situations are especially interesting. First, there is no report of a pre-prophase band in isolated cells in suspension culture (e.g. Roberts and Northcote, 1970), though perhaps the pre-prophase stage has not adequately been examined. Second, Wada and O'Brien (1975) found no band preceding a transverse division in a filamentous fern protonema, although pre-prophase stages were investigated.

In short, pre-prophase bands accurately predict the site of fusion of cell plates and parental walls, but they are not present in all cell divisions. Cell plates, including some that are placed asymmetrically, as in microspores and the Chara apex, can be inserted without their aid. The presently available information can, however, be rationalised if two inferences are drawn from the known distribution pattern.

One inference is that the band is concerned, at least in part, with the fusion of the cell plate and the parental wall. Thus, considering some of the negative situations, there is no parental wall in liquid endosperm preparations and there is no conventional cell plate after sperm mitosis. Cellularisation of endosperm in situ is by wall furrowing (in early stages) and where phragmoplasts do
participate (in later stages), their formation is not a consequence of preparations made at a prophase stage. The first division in microspores produces a cell plate, but instead of fusing with the parental wall as in (say) a root tip cell, the plate merely makes contact over a particular expanse of the parental intine, and the generative cell may detach later to become free-floating within the vegetative cell.

The second inference relates to those situations where cell plates and parental walls do fuse conventionally, but where the band is nevertheless absent. Bands are present in parenchymatous tissues but not (according to limited data) in filamentous organizations or single cells. Why should a file of cells in a fern protonema behave differently from a file of cells in, for example, an *Azolla* root? One obvious difference is that in the latter case the dividing cells are open to morphogenetic influences emanating from the surrounding cells. It seems reasonable to suggest, following Pickett-Heaps and Northcote (1966b), that the formation of an appropriately located preprophase band is a response to an intercellular signalling system, and further, that this system, and hence the band, is present where the complex architecture of two- and three-dimensional arrays of cells demands intercellular coordination of genetically controlled morphogenesis to a degree that is not found in single cells or filaments.
5.3.3 Formation of Pre-prophase Bands

The pre-prophase band cannot itself be causal in determining cell polarity or the plane of division. Nothing that is known about tubulin and microtubules suggests that they possess inherent information enabling them to be causal agents. There is, however, abundant evidence that they are versatile tools, deployed by the cell as and when required. Some of the most convincing evidence that intercellular signals specify the position to be occupied by the pre-prophase band derives from stomatal complexes in wheat, as described by Pickett-Heaps (1969a). The epidermal cells that give rise to the subsidiary cells do not have a standard developmental program to follow, but instead respond variously depending on the position and orientation of their end walls in relation to the guard mother cell: only the overall shape of the stomatal complex is specified, as by a gradient which can act at any appropriate location along one epidermal cell, or alternatively by spanning the adjoining ends of two epidermal cells. The postulated gradient could emanate from the guard mother cell (Pickett-Heaps and Northcote, 1966c; Pickett-Heaps, 1969a,b,c).

Stomatal complexes vary, and they represent but one of a very large number of distinctive cell patterns found in plants. The sources of positional information are unlikely to be the same in all cases. In the Azolla root new longitudinal walls are inserted
accurately in relation to the positions of the same categories of wall in the preceding merophyte (Figs. 5.15-5.18). Transverse walls are also inserted with precision (Figs. 5.1-5.3), the relative positions of the walls across the root being characteristic for each wall junction. In *Azolla*, nearly all new walls conform to the generalisation (Sinnott and Bloch, 1941) that the line of insertion avoids previously established wall junctions. Sinnott and Block (1941) noted exceptions where walls in adjacent cells were inserted in direct continuity with one another, and the outer tangential wall of the endodermis in *Azolla*, delimiting the stele, also shows no, or very slight, displacement. In general these displacements are only very slight, be they along lines of longitudinal or transverse walls, although even slight displacements in these latter walls will be exaggerated in older merophytes by the root elongation. However, in some situations, for example the positions of the transverse walls following the first proliferative divisions of the inner and outer cortex cells, quite large displacements are involved. In each case, the displacement, whether large or small, is accurately anticipated by the position of the pre-prophase band.

The position for the formation of the pre-prophase band thus may be determined by the interplay of intra- and intercellular signals. In terms of the mechanism of the formation of the pre-prophase band, many possibilities exist. Re-deployment of
interphase cortical microtubules to form the pre-prophase band is insensitive to cycloheximide (Benbadis et al., 1974 - evidence that a common tubulin pool is used). Pre-prophase band microtubules resemble those in interphase cortical arrays (Chapter 3) and perhaps the simplest form of instruction for re-deployment might be to alter the tubulin: microtubule equilibrium in favour of disassembly, except in a specified zone. Microtubules would then accumulate in this zone, generating the pre-prophase band.

In some cases (e.g. the transverse divisions in Azolla roots) the interphase cortical microtubules and the pre-prophase band microtubules share the same orientation, but in others this is not the case (e.g. the longitudinal divisions in Azolla [Fig. 5.16]). The present observations on variation in the numbers of microtubules in different regions of single bands, combined with microtubule maps obtained by serial sectioning (Figs. 3.15, 3.18) suggest that band development is non-uniform over the surface of the cell. There is no evidence (see Section 5.2.3) that the asymmetry in numbers of microtubules persists, or that the mature, functional band is other than a uniformly dense band of microtubules encircling the cell at the future site of cytokinesis.

Schnepf (1973) reported that the pre-prophase band was present for a longer fraction of the cell cycle than the phase of mitosis. His conclusion is
supported by the present data. In a sample of 4
*Azolla* root tips where every cell in the formative
zones was examined, pre-prophase bands were 13 times
more common than post-prophase mitotic stages. The
dermatogen cell layer also provides evidence for a much
longer duration of the band than of mitosis (Fig. 5.7).

The total length of microtubules in the pre-
prophase band in *Sphagnum* leaflet cells has been
estimated to be equal to or slightly greater than the
total length of the interphase microtubules (Schnepf,
1973). The data presented here, for *Azolla*, for the
pre-prophase band preceding the asymmetrical division
in the dermatogen (Fig. 5.7) and for the pre-prophase
bands preceding the first and second proliferative
divisions in the endodermis (Fig. 4.7) also reflect
a similar trend. These data not only support the
mode of formation suggested above, but may also account
for the different sizes of pre-prophase bands observed
in *Azolla*, as in other systems (Deysson and Benbadis,
1968).

The constant feature of the band is its width,
approximately 1.5-3µm, the degree of stacking of the
microtubules increasing as the number of microtubules
in the band increases (Fig. 3.18). The analysis of
microtubule distributions described in the preceding
Chapter (Section 4.3.2) showed that interpolation of
microtubules maintained a relatively constant dis-
tribution of interphase cortical microtubules in the
presence of rapid cell elongation. Thus, longer cells
contain a greater total length of microtubules than do shorter cells. Disassembly of the interphase microtubules in all regions except that in the specified zone of the future pre-prophase band, would lead to larger and more highly stacked bands in the larger cells.

5.3.4 Function of Pre-prophase Bands

All of the metaphase plates that have been seen in *Azolla* roots were correctly aligned at the site of, and in the plane of, the future dividing wall. It is also unlikely that the pre-prophase band functions in the migration of the nucleus to its site of division. In *Azolla*, nuclear migration is conspicuous only in the "one third" division (Fig. 2.46), and there, other microtubules may be involved, disappearing when migration is complete. In stomatal complexes, nuclear migration can precede pre-prophase band formation (Pickett-Heaps, 1969c), though the reverse sequence is seen in *Azolla*. Presumably the fundamental process is the polarisation of the cell. Pre-prophase band formation is one result of polarisation, and whether it occurs before or after nuclear migration may be trivial.

The *Azolla* root provides a spectrum of cell shapes and planes of division, and it may be significant that in precisely anticipating the future sites of cell plate fusion with the parental wall, the pre-prophase band microtubules are often much more
remote from the position where the nucleus will divide than might be expected to be optimal if the band is merely a precursor for the developing spindle.

Benbadis et al. (1974) show that a treatment with cycloheximide, that inhibits leucine incorporation into Allium root tips by 75-85%, does not prevent pre-prophase band formation, nor the laying down of some clear zone microtubules, but does prevent the transition from prophase to metaphase, and hence the development of the spindle apparatus. Unfortunately, it is not stated whether the pre-prophase band persists in inhibited cells; further such experiments might show if there is a precursor-product relationship between the pre-prophase band and the spindle, and whether tubulin molecules or intact microtubules, or both, are utilised.

Throughout the Azolla root, the position of the pre-prophase band anticipates with great accuracy where the new cell wall will join the old. The Azolla results thus agree fully with the high degree of predictability found by Pickett-Heaps and Northcote (1966b) and Pickett-Heaps (1969a,b,c) for stomatal complexes and with the observations of Gunning, Hardham and Hughes (1978a) on asymmetrical trichoblast divisions in Hydrocharis. Taken together with the absence of pre-prophase bands where cell plates do not form or do not fuse conventionally with existing walls (above), this consistent property of
predicting where cell division will be completed strongly suggests that a major function of the prophase band is concerned with at least the final stages of cell plate alignment and/or fusion with parental walls.

The only report at variance with precise spatial anticipation of the plane and site of division is that of Burgess and Northcote (1967) who state, for *Phleum* roots, that "the pre-prophase band....is placed symmetrically round the nucleus although a great many of the cells divide asymmetrically". A re-statement (Burgess, 1970b) is stronger: "Burgess and Northcote, 1967, 1968, demonstrated....that it was possible for the position of the band of microtubules on the mother cell wall to differ markedly from the positions at which the cell plate would fuse with it". If this observation is valid, it is crucial to any functional interpretation of the pre-prophase band. The asymmetrical divisions in question are those in the dermatogen, delimiting trichoblasts from atrichoblasts. They are not as markedly asymmetric as their counterparts in *Azolla* or *Hydrocharis* (Avers, 1963), most of the asymmetry appearing after division (Sinnott, 1960). Burgess and Northcote (1967) do not specifically state that they examined pre-prophase bands for this division, and in view of the asymmetry of the band in the equivalent division in two other species the point should perhaps be reinvestigated.
If the pre-prophase band functions late in cell plate development, why is it not formed during, or why does it not persist until, late telophase? Stebbins and Jain (1960) and Stebbins and Shan (1960) discuss the interaction of exogenous morphogenetic signals with gene-cytoplasm systems in receptor cells in order to regulate sites of cytokinesis. Species-specificity of cell division patterns is seen in *Azolla* roots just as in other situations (Gunning, Hughes and Hardham, 1978), and a requirement for transcription of specific endogenous genetic information may underlie the early development of the band. Mitchison (1971) and Prescott (1976) review the abundant evidence that transcriptional activity ceases (or is much inhibited) when the chromatin condenses at prophase of mitosis. It is therefore likely that any transcription that constitutes part of the overall control system for cytokinesis will have to occur prior to prophase, and the transcribed information must survive in some form until cytokinesis. The observed events suggest that translation of the information, in conjunction with exogenous signals of unknown nature, creates a specialised zone of cell cortex, recognisable to the edges of the cell plate or to the system that orients it (Palevitz and Hepler, 1974a,b). Whether the pre-prophase band results from this specialisation or functions in creating it remains an open question. It remains to discuss the specialisation that
is suggested to be conferred upon the cell cortex at the position of the pre-prophase band. Continuation or modification of wall deposition under the influence of the band microtubules is one possibility (Packard and Stack, 1976). However, these authors' suggestion that wall development becomes concentrated in the site of the band, eventually giving "H"-shaped pieces of new cell wall, is not supported by the observations made in *Azolla*. This process would have profound effects (that are not observed) on the distribution of plasmodesmata, and would also require secondary insertion of plasmodesmata into the growing arms of the "H". Secondary insertion can occur, but does not appear to be as general as the Packard and Stack model would predict (Jones, 1976); it has not been detected in *Azolla* roots (Gunning, 1978).

Reference is given to an extension of the idea (Palevitz and Hepler, 1974a) that pre-prophase bands "result from and thus indicate a morphogenetically important cortical initiating site.....(which) may remain and possibly control a subsequent formation of tubules along the plasmalemma during late division, which then may be able to influence the direction of growth of the centrifugally expanding cell plate and phragmoplast".

It is known from very many electron micrographs of *Azolla* roots that the site of cell plate fusion is specified (in relation to pre-existing walls) to a fraction of a micrometre, yet the
pre-prophase band is 1.5-3\mu m in width, straddling the future site of fusion. At first sight it would appear from this discrepancy that the band is too crude an instrument for the high resolution alignment that the Azolla system allows to be recognised. There is no gaussian or other distribution of microtubules in the band to imply that its midline is any different from its flanks. Nevertheless in Azolla the cell plate does fuse with the parental wall at or close to the midline of the band. Evidence concerning the mechanism of placement of the extreme edge of the cell plate is given in Gunning, Hardham and Hughes (1978b), meanwhile it is relevant to note that because the pre-prophase band zone is wider than the actual line of fusion, and becomes bisected, there is, in effect, a form of cytoplasmic inheritance, each daughter cell receiving half of the specialised cortical zone where the pre-prophase band was formed earlier in the cell cycle.

The working hypothesis that derives from the above observations and consideration of the literature can be summarised as follows. Pre-prophase bands of microtubules form in response to the combined effect of endogenous genetic information and exogenous morphogenetic signals, which, in two- and three-dimensional arrays of cells, participates in specifying planes of cytokinesis in categories of cell division where a cell plate is formed and fuses with the parental walls. The necessary genetic
transcription occurs before prophase condensation of chromatin, and the information is preserved in the form of a 1.5-3\(\mu\)m wide specialised zone of cell cortex at which microtubules accumulate temporarily before the spindle develops. The edges of the cell plate are later brought to the midline of the specialised zone and each daughter cell receives part of the former pre-prophase band region of the cell cortex.
Figs. 5.1-5.6  Pre-prophase bands for the formative atrichoblast-trichoblast division and for transverse divisions in the dermatogen, outer cortex and pericycle. The positions, in the root, of the cells containing the pre-prophase bands illustrated in the figures, are indicated in the light microscope section in Fig. 5.3.

Fig. 5.1  Two pre-prophase bands (bracketed), in adjacent dermatogen cells, for the asymmetrical, atrichoblast-trichoblast division. The wall cut in grazing section is the radial, longitudinal wall between the two dermatogen cells. X20,000.

Fig. 5.2  Two pre-prophase bands (black brackets), in adjacent dermatogen cells, for the second proliferative division. Again, the wall cut in grazing section is the radial, longitudinal wall between the two dermatogen cells. The white bracket encompasses the pre-prophase band for the second proliferative division along the outer tangential, longitudinal wall in the outer cortex. X11,000.

Fig. 5.3  A longitudinal, light microscope section of an Azolla pinnata root. X850.

Figs. 5.4-5.6  Two sections through the pre-prophase band for the second proliferative division in the pericycle. Fig. 5.4 shows the band near a corner of the cell. Figs. 5.5 and 5.6 show the band along the two, opposite walls two ultra-thin sections after that in Fig. 5.4. Fig. 5.4, X43,000; Figs. 5.5, 5.6, X50,000.
**Fig. 5.7** Pre-prophase bands for the asymmetrical atrichoblast-trichoblast division. Numbers 1 to 11 along the horizontal axes refer to a file of dermatogen cells in a longitudinal section of a root, the orientation being the same as that in Fig. 5.3. Cell number 1 is adjacent to the first dermatogen cell which has completed the asymmetrical division.

**Fig. 5.7A** Diagram showing the microtubule distribution along the central portion of the basiscopic, transverse wall in the dermatogen cells. Microtubule profiles are represented by dots and short lines. The distribution in cells 1 to 3 in a section approximately 10 ultra-thin sections away from the first, is also shown.

**Fig. 5.7B** The total number of microtubule profiles along the basiscopic (solid symbols) and acroscopic transverse walls (open symbols) along the file of cells.
**Figs. 5.8 - 5.12** Pre-prophase bands for division of the apical cell and the first tangential division.

**Fig. 5.8** Section cut transversely through the apical cell of a root which possesses clockwise segmentation. The oldest face of the apical cell is that on the left-hand side of the micrograph. The next division will occur at this face. X3,000.

**Figs. 5.9-5.11** The pre-prophase band in the apical cell in a longitudinal section of a root lying against the distal (curved) wall (Fig. 5.9) and the opposite triangularly-shaped face (Fig. 5.10). The subsequent cell plate shown in another root (Fig. 5.11) would have joined the parent wall approximately in the centre of the zone occupied by the pre-prophase band. Figs. 5.9, 5.10, X30,000; Fig. 5.11, X5,000.

**Fig. 5.12** The pre-prophase band for the first tangential division (division 1, insert) in a merophyte, in a longitudinal section of a root. Vesicles and dark-staining material are evident amongst the microtubules in the band. The walls shown are the acroscopic and basiscopic faces of the merophyte (see insert). X33,000.
Fig. 5.13-5.19 The formative divisions along the inner cortex and endodermal branches of the cell lineage diagram (Fig. 2.47).

Fig. 5.13-5.15 The upper edge of the figures points towards the apex, and the left-hand wall is that along the centre of the root. The plane of section coincides with the plane of the pre-prophase band (small arrows) for the radial, longitudinal division (number 3, see insert). The cell in the adjacent, basiscopic merophyte has formed the pre-prophase band for the next division in the sequence (number 5, see insert), a tangential, longitudinal division. This band is shown along the acroscopic wall in Figs. 5.13 (bracket) and 5.14 and along the basiscopic wall of the cell in Fig. 5.15. The cell in the adjacent basiscopic merophyte in Fig. 5.15 has completed division 5 and the midline of the pre-prophase band is positioned just to the left of the line of the longitudinal wall in the older merophyte. This displacement is comparable to that of the equivalent wall junction in the top, left-hand corner of Fig. 5.16. Vesicles and electron-dense material can be seen associated with the microtubules in the band. Fig. 5.13, X14,500; Figs. 5.14, 5.15, X60,000.

Figs. 5.16-5.18 The final formative division (number 7, see insert) is also in the tangential, longitudinal plane. The pre-prophase band for this division is shown along the acroscopic (Figs. 5.16, 5.17) and along the basiscopic wall (Figs. 5.16, 5.18) of the cells. Again, vesicles and matrix material are associated with the microtubules in the band. Fig. 5.16, X13,000; Figs. 5.17, 5.18, X30,000.

Fig. 5.19 A transverse section showing the first merophyte in a root, to have completed the formative divisions leading to the production of two inner cortex initials (large arrows) and two endodermal initial cells (small arrows). The other two merophytes at this level of the root, have yet to complete the final tangential, longitudinal division. X750.
CHAPTER 6
PRELIMINARY EXPERIMENTAL STUDIES
ON DEVELOPMENT IN AZOLLA PINNATA ROOTS

6.1 INTRODUCTION

Early studies using the drug colchicine (see Eigsti and Dustin, 1955), showed it to be a potent inhibitor of mitosis in both plant and animal cells. Mitosis is arrested at metaphase and cells accumulate at this stage of the cell cycle. It was at first thought not to have any effect on cells in prophase (Levan, 1938) but was later shown to sometimes induce reversion of partially condensed chromosomes back to the dispersed interphase form (Eigsti and Dustin, 1955). In cells at metaphase, colchicine treatment leads to the partial or total destruction of the spindle fibres, the metaphase orientation of the chromosomes is lost and the chromosomes assume various forms of clusters. Due to the loss of the spindle fibres, normal chromosome separation at anaphase is not possible although they do sometimes separate in a disorganised fashion, the chromatin dispersing and two or more "restitution" nuclei forming. Both plant and animal cells can recover after colchicine treatment and recent experimental studies have utilized the colchicine-induced metaphase accumulation to estimate cell cycle durations in plant roots (Clowes, 1975a, 1976).
When roots are immersed in colchicine solutions elongation ceases and the root tips swell forming "colchicine-tumours" (C-tumours), caused by an increase in volume of the meristematic cells without cell division or elongation (Levan, 1938; Brennan, 1970; Stein, 1971). Growth of roots in deuterium oxide has also been reported to induce lateral swelling of root tips (Stein et al., 1971).

After spindle fibres had been equated with bundles of cytoplasmic microtubules, it was realised that colchicine treatment caused the destruction of these microtubules. In plant cells, cortical microtubules adjacent to primary walls during interphase and pre-prophase and those overlying zones of secondary wall deposition in differentiating xylem cells were also found to disappear following colchicine treatment (Pickett-Heaps, 1967). In this latter case, loss of the cortical microtubules leads to irregularly shaped and distributed masses of secondary wall. During the deposition of both primary (Green, 1969) and secondary wall material (Hepler and Fosket, 1971) the loss of microtubules is associated with loss of oriented cellulose microfibril deposition. Studies of the effects of colchicine on the roots of *Phleum* suggested that the lateral cell expansion causing C-tumour formation is primarily due to the removal of the cortical microtubules and the consequent loss of transverse microfibril orientation (Brennan, 1970). C-tumour formation, but not metaphase arrest, has been noted in a number of fern species (Chiang and Lu, 1972). The importance of cortical microtubules in morphogenetic processes in plant cells has been emphasised in the
preceding Chapters. However, the question of how these highly organised arrays are set up has not been examined. Physical and chemical treatments which affect microtubule systems are likely to be helpful in this regard, as they have been in the determination of microtubule functions. Morphological and ultrastructural effects of a variety of treatments with colchicine and other chemical and physical agents on Azolla pinnata roots are described in this Chapter. Once again, only the very precise knowledge of the construction of the A. pinnata root described in Chapter 2 has made the following experimental studies possible.

The Materials and Methods used are as previously described or as detailed in the Results Section (6.2).

6.2 Results

6.2.1 Low Temperature, High Pressure and Isopropyl N-Phenylcarbamate

Low temperature and high pressure treatments were found to affect the structure of cortical microtubule arrays in Azolla pinnata root tip cells (Section 3.3.7). A 15 minute or 4 hour treatment at a temperature less than 2°C or 15 minutes at 14,000 or 16,000 psi reduced the average microtubule length to less than half the normal value. 15 minutes at room temperature after a low temperature treatment allowed a rapid recovery of the average microtubule lengths to that of controls. Measurement of the root lengths of plants grown in alternating periods of low
temperature (2°C) and normal temperatures (12-15°C) showed that root elongation ceased at the low temperatures but rapidly resumed at normal rates at the warmer temperatures (Fig. 6.1).

To be certain of the exact effects of these treatments on the microtubule arrays information on the numbers of microtubules present as well as the lengths is necessary. Knowledge of the pattern of microtubule distribution in proliferating endodermal cells in untreated roots (Section 4.3.2) enables a comparison to be made between the normal microtubule distribution and that resulting in the roots after the treatments which affect the cortical microtubules. The results of counts of microtubules along files of endodermal cells in roots fixed after 2 hours at 0°C and 15 minutes at 14,000 psi are shown in Figs. 6.2A and B respectively. Comparison with control curves (Fig. 4.7) shows that the frequencies of microtubules along both the inner and outer tangential walls has been reduced, particularly after high pressure treatment.

Isopropyl N-phenylcarbamate (IPC) is thought to disrupt microtubule arrays by affecting the MTOCs. Solutions of 5 x 10⁻³M IPC in culture medium completely inhibited the growth of *Azolla pinnata* plants measured by daily photographic record. In another experiment, a number of plants was transferred to culture medium containing IPC at 0°C for 30 minutes and then allowed to recover in fresh
medium at 20°C for 12, 24 and 36 hours. No ab-
normal effects on root structure or xylem
differentiation was evident in cleared roots after
these recovery periods.

6.2.2 Deuterium Oxide

Although the effects of growth in pure D₂O
have only been measured for a limited number of
plants, a general cessation of root elongation
accompanied by swelling of the roots, particularly
those less than approximately 1.0mm in length, is
indicated by the measurements that have been made.
The very stunted appearance of roots greater than
1.0mm in length is exemplified in Figs. 6.3-6.5. The
root cap and root hairs are much shorter than
normal and there are only 1 or 2 epidermal cells
between adjacent root hairs instead of the 4 or more
seen in the untreated root of comparable length
(Fig. 6.6).

The number of microtubules in the endodermal
cells in roots immersed in D₂O for 5 hours appears
very similar to control values (Fig. 6.2D). After
18 hours (Fig. 6.2E) the number of microtubules along
the inner tangential wall in the younger cells may be
greater than in untreated roots but the numbers along
the outer tangential wall appear slightly reduced.
The data are, however, limited and more counts are
required before any definite trends can be ascertained.
6.2.3 Long Term Colchicine Treatments

Growth of *Azolla pinnata* plants in solutions of culture medium containing $5 \times 10^{-3}$M colchicine resulted in the inhibition of growth of both shoots and roots. Daily measurement of the lengths of young roots revealed there to be very little elongation (Fig. 6.15A). After 2 days in the colchicine solution lateral expansion of the roots became evident and continued until the roots were approximately twice the width of untreated roots of the same length (Fig. 6.7, 6.15C). An inhibition of root hair elongation was also noted (Fig. 6.8).

Continuous colchicine treatment resulted in a decrease in the number of merophytes in the roots compared to control roots (Fig. 6.15B). After 1 day in the colchicine solution, the acropetal progression of xylem differentiation is retarded in some roots, but after 2-3 days, in the absence of root elongation (Fig. 6.15A), xylem differentiation progresses closer to the apex than in untreated roots (Fig. 6.15D).

In the presence of colchicine, secondary wall deposition in the xylem is highly irregular (Figs. 6.9, 6.12, 6.13). Discrete rings do not form, but isolated patches of secondary wall material are deposited all over the three longitudinal cell walls. Although many roots did not contain any elements with abnormal secondary wall deposition, in other roots, from 1-5 elements along a file were affected. Based on the analysis of xylem differentiation in
untreated roots (see Section 4.3.3) this difference in the number of affected cells seemed likely to be dependent on the length of the root at the commencement of treatment. This appears to be the case, as roots between 0.25mm to 0.35mm in length, i.e. those in which xylem differentiation is progressing acropetally at a rapid rate, show the maximum effect.

No microtubules were observed in roots fixed after 24 hours in colchicine solutions, but a number of xylem elements in these roots possessed masses of electron dense amorphous material overlying areas of secondary wall deposition (Fig. 6.14).

6.2.4 Short Term Colchicine Pulse Treatments

A pulse time of colchicine was sought which would elicit the effect on xylem differentiation described in the previous Section and also allow cell development to recover after removal of the drug. 24 hour and 10 hour pulses affected xylem differentiation to the same degree as the continuous colchicine treatment, i.e. varying numbers of elements were affected, depending on the root length, but no recovery of regular secondary wall deposition in the xylem was obtained (up to 3 days after treatment) [Figs. 6.9-6.13]. Seven, 5 and 2 hour treatments, however, were found to fulfil these two criteria (Figs. 6.23-6.26). The average microtubule
length following a 2 hour or 5 hour exposure of the
*A. pinnata* roots to colchicine solutions was deter-
mined to be less than half the control value (Table
3.7). Counts of microtubules in proliferating endo-
dermal cells after a 4 hour colchicine treatment
revealed that the numbers of microtubules were also
reduced (Fig. 6.2C). These shorter colchicine pulses
do not cause complete microtubule depolymerisation
during the treatment time but they are long enough
to influence cellular processes in the centre of the
root as exemplified by abnormal xylem wall deposition,
and thus complete penetration of the root is achieved.
The effects of the shorter pulse time (i.e. 2 hours)
on root and shoot growth and on cell division and
differentiation in the roots were then examined in
greater detail.

*Shoot and Root Growth:*

The 2 hour colchicine pulse was found to have
an inhibitory effect on both shoot and root growth.
Analysis of daily photographic record of the plants
showed that fewer new leaves appeared at the growing
shoot apices than in untreated plants. The effect
of the 2 hour colchicine pulse on root elongation
was not the same for all roots along a frond.
Measurement of the lengths of roots along a branch
after the pulse are shown in Fig. 6.16A and can be
compared with growth rates of control roots in Fig.
2.50. There is an immediate decrease in the elongation
rate of roots greater than approximately 0.7mm in length relative to control roots, becoming progressively more severe until, after 4-5 days, these roots have virtually ceased elongation. On the other hand, roots less than approximately 0.7mm in length at the time of treatment did not show an immediate decrease in growth rate, and in fact in the first 24 hours following treatment, some roots had rates which exceeded those of control roots of the equivalent lengths. However, after 4-5 days, growth had largely ceased in these roots also.

During this period the roots expanded laterally and after 8 days the widths of the roots were the same as those of roots grown in colchicine continuously for the same length of time (Figs. 6.12, 6.15C).

Cell Division and Cell Differentiation:

The number of merophytes in roots of different lengths after the 2 hour pulse followed by 12, 24 and 36 hour recovery periods was reduced as compared to values in untreated roots (Fig. 6.16B). The 2 hour pulse did not have a significant effect on the distance of the first xylem element with secondary wall deposition from the root apex after 24 or 36 hour recovery periods (Fig. 6.16C). Again, the number of affected elements along the xylem files depended on the root length at the time of treatment, with up to 5 elements along the xylem files showing abnormal wall deposition. The effects of the
colchicine treatment were not immediately evident and abnormal secondary deposition was not visible in the xylem until 12-24 hours after the colchicine pulse (Table 6.1). After a 36 hour recovery period a few, but not many, roots were found in which normal xylem thickening formation had resumed in cells closer to the apex than those in which abnormal wall deposition had occurred (Table 6.1). Only roots at very early stages of the phase of rapid xylem differentiation, i.e. approximately 0.2mm in length at the time of treatment, would be likely to exhibit a recovery. In some longer roots (approximately 1.0mm in length) examples of recent, normal wall deposition in the most acroscopic elements were found but where there were no affected elements, possibly indicating the complete absence of wall deposition in these roots in the presence of colchicine, with a resumption of normal wall deposition during the recovery period.

Twenty-four hours after the colchicine pulse, roots approximately 0.5mm in length show the greatest number of affected xylem elements - often all of the differentiated elements in the root. Details of the abnormal wall deposition as seen using both Nomarski interference-contrast optics and electron microscopy are shown in Figs. 6.17-6.22, 6.27, 6.28. The secondary wall has been deposited in localised areas but is irregular and no complete rings are formed. The normal bands of microtubules are not present but
in many cases clouds of vesicles and electron-dense material overlie the wall masses (Figs. 6.27, 6.28). A few microtubules are sometimes associated with these complexes, depending on the length of the root (see later).

A survey of cleared roots 36 hours after treatment revealed that recovery of normal secondary wall deposition could occur (Table 6.1). A limited number of roots contained xylem elements with regular secondary thickenings in positions acroscopic to elements in which secondary wall deposition had been disturbed (Figs. 6.23-6.26). An element at very early stages of this recovery has been found in embedded material. Clusters of vesicles and electron-dense material similar to those seen in the root after 24 hours recovery were seen in association with groups of transversely aligned microtubules (Fig. 6.29). These complexes occurred along an outer corner of a young element, 14-15 merophytes from the apex of a 0.47mm root. Using the growth curves in Fig. 2.51, a length of 0.2mm at the time of treatment was estimated.

Details of the presence or absence of microtubules and other ultrastructural features in roots of different lengths after different recovery times are given in Table 6.2. The recovery appears to be complex, the length of the root at the time of treatment being important. The data are limited but are sufficient to show a number of interesting points and possible trends.
Multinucleate Cells:

As well as irregular xylem differentiation, a multinucleate condition in the root apical cell was observed in cleared roots. Examination of sectioned material in the electron microscope confirmed this and also showed a greater than normal vacuolation of the apical and sub-apical cells (Figs. 6.30-6.33). Up to 7 nuclear profiles were seen in apical cells in ultra-thin sections of recovering roots (Figs. 6.30-6.33; Table 6.2). Up to 4 nuclear profiles were seen in other cell types in the root apex in cells undergoing formative or proliferative divisions (Figs. 6.35-6.37, 6.44). In many cases the cells were apparently binucleate, containing two large normal-size nuclear profiles. Examples of this situation were often seen in merophytes 1-3 in which there had been no division subsequent to the merophyte formation by division of the apical cell. The impression gained is one of nuclear division without subsequent cell plate formation due to the lack of phragmoplast microtubules. In other cases one or two normal-sized nuclear profiles were accompanied by smaller profiles (Figs. 6.35-6.37) or the nuclear profile was highly lobed. Cells arrested at the metaphase stage of mitosis were observed throughout the roots (Figs. 6.31, 6.36, 6.37), including a root cap cell.

Microtubules and Wall Formation:

The presence and absence of the microtubules
in the roots after the various recovery times (Table 6.2) was quite unexpected. Although microtubules were still present after the two hour treatment they had completely disappeared in at least some, if not all, roots subsequent to treatment (Fig. 6.38). The reappearance of the microtubules is not a purely time-dependent process as the length of the root at the time of treatment also seems to be involved. After 12 hours recovery, microtubules are abundant in the 0.53mm long root but totally absent from the shorter roots. During continued recovery periods the microtubule arrays progressively reappear in the roots which were shorter at the time of treatment (Fig. 6.39).

Twenty-four hours after treatment signs of new wall formation were seen in the roots 0.93 and 0.56mm in length in the form of fragments of wall in a number of dermatogen cells, which would normally have been completing their first proliferative division (Table 6.2). In the roots allowed to recover for 36 and 48 hours, new wall formation was evident in many cells throughout the roots. These new walls were distinct from the incomplete cell plates which remained after the loss of the microtubules (Fig. 6.40) and were identifiable by abnormal electron-dense inclusions in the wall material (Figs. 6.32-6.35, 6.40-6.46). The new wall formation was seen only in roots which had at least begun to reinstate their microtubule arrays (Table 6.2). In
many cases the new walls were complete and normally positioned in the parent cell (Figs. 6.35, 6.44, 6.45), although sometimes irregular in outline (Figs. 6.35, 6.44). Usually, however, the walls were incomplete and had only begun to subdivide the multinucleate cells. In some cases, for example the apical cells of the two shorter roots after 36 hours recovery (Figs. 6.32, 6.33), the wall fragments were isolated in the cytoplasm without contact with a parental wall, at least in the sections examined. In most cases, however, the wall fragments adjoined one parental wall and projected between the nuclei in the cells (Figs. 6.41, 6.42, 6.44). In the 0.47mm long root, after this recovery period, complete subdivision of the apical cell had occurred (Fig. 6.43). Complex anastomosing wall masses also occurred either attached to parental walls (Figs. 6.34, 6.46) or isolated in the cytoplasm (Fig. 6.44). These isolated wall masses may adjoin parental walls in other regions of the cell. Microtubules were often seen in association with these wall fragments (Fig. 6.34). Plasmodesmata were, in general, not abundant in the new walls, however unusual clustering of plasmodesmata is present in the wall mass in Fig. 6.34. The incomplete wall fragments, and the new walls which have completed subdivision of the cells, usually adjoin the parental walls at the normal sites of fusion of the cell plate. In the 24 and 36 hour-recovered roots many examples of binucleate and trinucleate
dermatogen cells, which had completed the second round of proliferative divisions, were seen. Figs. 6.41 and 6.45 show similar cells which have proceeded to subdivide in attempts to complete the oblique root hair division after 36 hours and 48 hours respectively. The new walls are attached to the correct location along the basiscopic transverse wall even though apparently the position of the nuclei, in some cells, did not allow normal positioning of the rest of the wall.

In the final examples to be considered, the wall fragments or the completed walls, although adjoining the parental walls at normal sites, did not do so at the locations which would lead to the correct plane of division in the normal sequence of divisions. In Fig. 6.42, the dermatogen-outer cortex precursor cell has begun to subdivide, but instead of forming the longitudinal wall which would normally complete the sequence of divisions along the dermatogen-outer cortex branch of the cell lineage, a transverse wall had begun to form.

A similar abnormality is shown by the completed walls in 2 of the 3 inner cortex-endodermis precursor cells in the left-hand cell file in the section in Fig. 6.44. These cells have completed formation of a transverse wall instead of the normal longitudinal wall which would have, again, completed the final formative division, yielding an inner cortex initial cell and an endodermis
initial cell. The position of the transverse wall in the third cell clearly shows the slight basi­scopic displacement characteristic of the inner cortex proliferative divisions.

6.3 DISCUSSION

6.3.1 Introduction

Having described the morphogenesis of *Azolla pinnata* roots (Chapter 2); the geometry of the cortical arrays of microtubules found in the root cells (Chapter 3); the patterns of microtubule frequency during the proliferative division and differentiation phases of growth in selected cell types (Chapter 4); and the anticipation of planes of division throughout the roots by pre-prophase bands of microtubules (Chapter 5); it is logical to progress towards experimentation on the roles of microtubules in morphogenesis. The present Chapter contains preliminary observations dealing with the effects of physical (low temperature, high pressure and D₂O) and chemical (IPC, colchicine) treatments that are known to affect microtubule systems.

6.3.2 IPC, Low Temperature, High Pressure and D₂O

IPC was found to disrupt the orientation of microtubules in the mitotic spindles of *Haemanthus* endosperm cells and it was suggested that the IPC might bind to the structurally amorphous spindle organising material, i.e. the microtubule nucleating
sites (Hepler and Jackson, 1969). The drug was subsequently used in investigations of cortical microtubule organisation in *Ochromonas* where the microtubule nucleating sites had been identified (Bouck and Brown, 1973; Brown and Bouck, 1974). In cells not regenerating their microtubule arrays, IPC had only a slight effect on cytoplasmic microtubules and cell shape. However, after simultaneous application of IPC and high hydrostatic pressure the cells became spherical and the cytoplasmic microtubules were transformed to larger diameter macrotubules. In cells recovering from IPC-pressure treatment the macrotubules disappeared and microtubules reappeared at the same nucleating sites concomitant with the reappearance of normal cell shape. This recovery was independent of new protein synthesis. In other plant tissues, when used alone, IPC leads to a reduction in microtubule numbers in *Allium* roots (Brower and Hepler, 1976) or guard cells (Palevitz and Hepler, 1976) or virtual elimination of cortical microtubules in *Triticum* roots (Brower and Hepler, 1976). The treatment resulted in loss of secondary wall pattern in the xylem in these roots. In the developing guard cells the wall thickenings lost their birefringence, often becoming abnormally segmented into discrete sub-thickenings, and multiple pores were formed.

If IPC acts at the level of the MTOCs, then application of this drug alone would only lead to
partial microtubule disruption in a cell in interphase, due to the possible low rate of microtubule formation. Much greater disruption of cortical microtubules would be expected in a cell that was re-establishing its microtubule arrays after either high pressure or low temperature treatments (which do not involve a requirement for new protein synthesis). It was hoped that 30 minutes low temperature treatment of *Azolla* roots in the presence of IPC would cause a disorganised formation of microtubule arrays, culminating (for example) in abnormal xylem thickening deposition, but this was not observed. Possibly a longer time at the low temperature is needed, or a better approach may be to depolymerise the microtubules using high pressures as in the work on *Ochromonas* (Brown and Bouck, 1974). Although 15 minutes at 0°C or 15 minutes at 14,000 or 16,000 psi reduced the average microtubule length to approximately one half the normal value (Table 3.7), the estimates of relative microtubule numbers presented in this Chapter (Figs. 6.2A,B) show there to be a greater reduction in number after the 14,000 psi treatment.

The preliminary experiments with low temperature, D₂O, IPC, and high pressure revealed little, but served to emphasise the need to design experiments that would yield interpretable results. What was clearly needed was something between the total inhibition of growth given by continuous D₂O and IPC
treatments, and the lack of effectiveness of the combined IPC and low temperature pulse treatments. Accordingly, attention was focused on one type of perturbation, namely treatment with colchicine. Emphasis was placed on devising a sub-lethal treatment that would be effective, but from which the roots could recover. It was also considered important to collect background information on growth and development in the system, so that the ultrastructural observations could be interpreted meaningfully.

6.3.3 Colchicine

C-tumour Formation and Inhibition of Root Elongation:

A variety of colchicine treatments have been found to induce tumour formation in a number of angiosperms and fern roots due to cessation of root elongation and the continued increase in volume of the meristematic cells in the absence of cell elongation or division. Continuous colchicine treatment produces tumours in *Allium* after 5 days in an 0.1% solution (Levan, 1938); in *Phleum* after 17-24 hours in 0.02-0.05% solution (Brennan, 1970); in *Zea* after 8 hours in a 0.1% solution (Stein, 1971); and in the ferns, *Ceratopteris, Ophioglossum* and *Marsilea*, after 48 hours in a 0.05% solution (Chiang and Lu, 1972), and in *Azolla* after 3 days in a 0.2% solution (present work).
Continuous colchicine treatments are not necessary to produce C-tumour formation. 10-15 minutes in a 0.1-0.2% colchicine solution induces tumour formation in *Phleum*, visible 24 hours later (Brennan, 1970); a 3 hour treatment of *Pisum* roots with 0.025% colchicine results in tumour formation 18 hours after treatment (Barlow, 1969). In *Azolla*, a 2 hour pulse of 0.2% colchicine produced tumours after 8 days "recovery", the dimensions of those caused by continuous colchicine treatment. Both continuous and pulse treatments in *Azolla* increased the root width to approximately twice that of control roots (Fig. 6.16C). A similar increase (1.8-2.5-fold) was observed in *Ceratopteris*, *Ophioglossum* and *Marsilea* (Chiang and Lu, 1972).

Following the 2 hour colchicine pulse, root elongation was inhibited to varying degrees, depending on the length of the root at the time of treatment (Fig. 6.16A). Differential effects of colchicine treatments on roots at different stages of development have also been reported in *Vicia faba* (Davidson and MacLeod, 1968; Davidson, 1969; MacLeod, 1976b). Primary and lateral roots and small primordia were affected by colchicine treatment but large primordia were not, and did not form tumours (Davidson and MacLeod, 1968; Davidson, 1969). It has been suggested that this difference may be due to lack of colchicine penetration in some primordia (D'Amato and Nuti Ronchi, 1968; MacLeod,
The studies of the *Azolla* roots following colchicine treatment indicate that this may not necessarily be the cause of the differential responses. Effective colchicine penetration in roots of all lengths during the 2 hour treatment is indicated by the abnormal secondary wall deposition in the xylem at the centre of the roots but different growth responses are still observed for roots of different lengths.

The 2 hour colchicine pulse ultimately results in the cessation of growth of all *Azolla* roots. In contrast, complete recovery of root growth has been observed in *Allium* (Levan, 1938); *Pisum* after 3 days (Barlow, 1969); and in *Zea* after 1 day (Stein, 1971). In *Vicia* primordia may either die, have their development inhibited, grow as straight laterals or form C-tumours and emerge as inhibited laterals, depending on the stage of development of the primordium at the time of treatment (MacLeod, 1976b). MacLeod (1971) correlated the recovery of root proliferating tissues from colchicine-induced inhibition of growth, with the presence of quiescent cells. Meristems which consist solely of proliferating cells do not recover from colchicine treatment, while meristems which contain many quiescent cells recover more rapidly than those which contain fewer. Previous studies of colchicine effects on fern roots (Chiang and Lu, 1972) did not incorporate recovery periods but the behaviour of
Azolla roots reported here, supports this suggested role for the quiescent centre cells which do not exist in the fern roots (see Section 2.4.3). The roots lose their capacity for elongation, probably due to the loss of transverse alignment of the wall microfibrils following colchicine-induced destruction of the cortical microtubules. In angiosperm roots the quiescent centre supplies new initial cells, which can bring about resumption of growth at the apex. Without a quiescent centre, fern roots are unable to recover.

**Ultrastructural Effects**

Colchicine destruction of microtubules and consequent arrest of mitosis at metaphase is used in studies, mainly in angiosperm roots, of mitosis and cell cycling. Cortical microtubules are also destroyed following colchicine treatment and the drug has been employed in investigations of cortical microtubule functions, whether they be cytoskeletal, or involved in both primary and secondary wall formation. In the former studies, recovery periods ranging from 12 hours to 3 days have been needed before normal mitosis and root growth resumes. Only a few studies of wall formation have included recovery periods following colchicine treatment. In Pisum roots, apical growth resumed 3 days after a 3 hour treatment and it was noted that a recovery of protoxylem differentiation but not metaxylem differentiation occurred (Barlow, 1969). The other three
examples of recovery of normal wall formation are in the algae, *Nitella* (Green, 1969); *Oocystis* (Grimm *et al.*, 1976); and *Ochromonas* (Brown and Bouck, 1973). In *Nitella*, removal of colchicine allows the resumption of transverse cellulose deposition. In *Oocystis* a recovery in the normal alternation of lamellae could be obtained if colchicine treatment lasted no more than 24 hours. After a 5 hour colchicine treatment, in *Ochromonas*, shape regeneration commenced 3 hours after removal of the colchicine and in 10 hours the cells had completely recovered. This recovery after colchicine treatment did not occur in the presence of cycloheximide, indicating the requirement of new protein synthesis for the reformation of the cortical microtubules.

Although growth of *Azolla* roots does not recover after the 2 hour colchicine treatment (possibly due to the absence of a quiescent centre, as discussed above), normal secondary wall deposition in the xylem does resume about 36 hours after treatment. There must therefore be some degree of recovery of normal activity in at least some cells. Examination of Table 6.2 shows that this recovery is not straight-forward but appears to involve differential rates of recovery of microtubules and/or rates of destruction of microtubules, depending on the stage of development of the root at the time of treatment. An attempt to summarise the possible trends during recovery is set out in the flow diagram below.
2 HOUR COLCHICINE

Average microtubule length approximately half control values. Number of microtubules reduced.

12 HOUR RECOVERY

Abnormal secondary wall deposition visible in most acroscopic xylem elements. Cells arrested at metaphase and multinucleate cells present throughout roots. Microtubules abundant in root 0.53mm in length but completely absent in 0.25mm root.

24 HOUR RECOVERY

Further abnormal secondary wall deposition in xylem. Microtubules abundant in 0.93mm root, present in 0.56mm root but absent in 0.2mm root. Wall formation in dermatogen cells.

36 HOUR RECOVERY

Normal secondary wall deposition in acroscopic xylem elements. Microtubules abundant in roots ≥0.47mm, and present in roots 0.17mm and 0.32mm in length. Wall formation in multinucleate cells throughout roots ≤0.47mm in length - includes complete subdivision of the apical cell in the 0.47mm root, and partial wall formation in the apical cells of the two shorter roots.

48 HOUR RECOVERY

Microtubules present in 0.57mm root but absent from 0.27mm root. Wall formation throughout 0.57mm root.
Most interpretations of these data can only be speculative but they do contain a number of definite results:

1. Colchicine treatment can lead to the formation of multinucleate cells and the arrest of mitosis at metaphase in *Azolla* roots.

2. Although microtubules were still present at the end of the 2 hour colchicine treatment, complete disappearance of microtubules can occur subsequent to the treatment.

3. Microtubule arrays can be re-established 36 hours after colchicine treatment.

4. Normal secondary wall deposition can resume approximately 36 hours after treatments.

5. Partitioning of multinucleate cells can occur after 24 hours of recovery.

The first of these results is contrary to the suggestion made by Chiang and Lu (1972) that meristematic cells of ferns differ from those in angiosperms in their response to colchicine by developing no C-metaphase nuclei. Although these authors found no arrested metaphase figures after a 48 hour colchicine treatment in *Ceratopteris*, *Ophioglossum* or *Marsilea*, multinucleate and amoeboid nuclei were seen.
A possible explanation for the second observation may be the persistence of the drug in the cells after transfer of the plants to fresh culture medium. Although able to penetrate relatively rapidly, the colchicine may not diffuse out of the cells but might remain to bind to any free tubulin, thus continuing to cause microtubule depolymerisation, until all binding sites are occupied or until the molecules are inactivated (Hart and Sabnis, 1976).

Reformation of the microtubule arrays after complete disassembly is indicated by the deposition of normal secondary thickenings after 36 hours in xylem elements closer to the apex than those in which abnormal deposition had occurred. Ultrastructural confirmation of the presence of microtubules was obtained for roots of comparable length at this time. Measurement of root growth following the 2 hour colchicine pulse showed that elongation continued at approximately normal rates for the first 24-48 hours for roots of lengths less than approximately 0.7mm. Assuming normal rates of elongation, approximate root lengths at the time of treatment can be estimated using the graph in Fig. 2.51. As expected, it is the roots at the very early stages of xylem differentiation, i.e. 0.15-0.25mm in length at the time of colchicine treatment, which show the recovery after 36 hours. The ultrastructural observations show that 12 hours after treatment a root 0.2mm in length completely lacks microtubules, but
36 hours later, correlated with the deposition of normal secondary thickening in the acroscopic xylem elements, cortical microtubules are again abundant.

The recovery of the microtubule arrays is complicated by an apparent difference in roots of different lengths. Either the microtubule arrays in the longer roots were not completely destroyed by the colchicine or the microtubules are destroyed in all roots, but reappear more rapidly in the longer roots. The former situation could perhaps arise if the drug did not penetrate the longer roots or if the microtubule arrays were more stable in these roots. Neither of these possibilities is at all likely. Lack of penetration is refuted by the effects on the xylem in these roots (and even in the stem above the roots). Before treatment, formative and proliferative divisions are occurring in these roots, the microtubule populations undergoing the normal regular formation and dissolution and a greater stability in these rapidly cycling cells would seem highly improbable. Indeed, the data provide support for the alternative explanation, i.e. that the microtubules are destroyed in all roots but reappear more quickly in the longer roots. Measurements of root growth showed that growth of the very short roots is slow, the rate of growth increasing as the root length increases. Using these data, calculation of apical cell cycle durations revealed that the apical cycle
time was also longer in the very short roots, becoming more rapid until a minimum time of 3-4 hours was reached in roots approximately 0.3mm in length. The analysis described in Chapter 2 also showed that many of the formative and proliferative divisions keep pace with the division of the apical cell.

These facts combine to give a picture of extremely active cell cycling in roots 0.3-0.7mm in length. It would seem likely that this rapid cycling would be reflected in a higher metabolic rate in the cells of these roots than in those of the shorter more slowly-growing roots with more slowly-dividing cells. In turn, this higher metabolic capacity may enable more rapid synthesis of new protein for the re-establishment of the microtubule arrays. The higher metabolic rate is not only reflected in the rate of appearance of microtubules but also in the appearance of new wall synthesis. After 24 hours recovery, the two longer roots which have at least begun to reinstate their microtubule arrays have also begun to subdivide their multinucleate cells. After 36 hours the 0.47mm root has completed the subdivision of its apical cells whereas the 2 shorter roots have only begun this process. Although incomplete wall profiles were seen with or without the association of microtubules, no wall formation is evident in roots which have not yet begun replacement of their microtubule arrays.
Subdivision of the multinucleate cells does not proceed by way of the normal phragmoplast and cell plate. Wall formation appears to be initiated at one parental wall and the expanding wall then grows across the cell between the two nuclei, finally fusing with a second parental wall. In nearly all cases the site of initiation was at the correct location for a new wall, be it for the formative division creating a trichoblast in a dermatogen cell, or the transverse, proliferative division in the dermatogen or cortex. Some of the completed walls also joined the second wall at the normal position but in other cases this was not possible, apparently due to the positioning of the nuclei or other cell components (e.g. the vacuole in the first merophyte in Fig. 6.35). The transverse divisions in the dermatogen-outer cortex precursor cell and in the inner cortex-epidermal cells were positioned correctly, not for the final longitudinal wall completing the formative division sequences, but for the first proliferative division of the dermatogen or outer cortex and the inner cortex respectively. Priority may be given to wall placement which is subject to controls that advance acropetally down the root, including the cell-by-cell progression of the asymmetric division in the dermatogen, and the transverse divisions which have been shown to be keyed to cell elongation (Chapter 2). It is relevant to consider the possible importance of the
stage of development of the cells at the time of drug treatment, in the context of these latter abnormalities. For example a cell that has reached the pre-prophase band stage may react differently to one at an earlier stage of development. Cells may be in a phase of "pre-commitment" to a particular plane of division as against a "post-commitment" phase. The cells in the above examples may have been in the pre-commitment stage to the final longitudinal formative division, enabling the factors associated with the regulation of the transverse divisions to influence the placement of the new walls.
Table 6.1 Two hour colchicine pulse and xylem secondary wall deposition.

<table>
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<th>Recovery period (hours)</th>
<th>Root length (mm)</th>
<th>Number of xylem elements along the two cell files:</th>
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<th>Affected</th>
<th>Recovered</th>
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<td>Recovery period (hours)</td>
<td>Root length (mm)*</td>
<td>Multinucleate cells:</td>
<td>Arrested Metaphase</td>
<td>Microtubules</td>
<td>Abnormal wall formation:</td>
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</table>

*The bracketed root lengths are the estimated lengths at the time of treatment.

†Wall formation has completely subdivided the 6 or more nuclei in this apical cell.
FIG. 6.1 Inhibition of root elongation by low temperatures.

No root elongation occurred when the temperature of the nutrient medium was lowered to approximately 2°C (shaded areas). On the return to warmer temperatures (12-15°C) root elongation resumed.
FIG. 6.2 The numbers of microtubule profiles per µm along the longitudinal walls in endodermal cells in plants which had been treated with physical and chemical agents which affect microtubules. These microtubule distributions may be compared to those in control roots in Fig. 4.7.
Figs. 6.3-6.14 The effects of D$_2$O and colchicine on root development.

Figs. 6.3-6.5 Three roots (large arrows) along a frond grown in pure D$_2$O. Root elongation has been inhibited and lateral swelling has occurred in the two shorter roots. The root tips are black and presumably dead and the root cap (brackets) is shorter than normal. The longest root (approximately 2mm in length) was cleared and observed using Nomarski interference-contrast optics (Figs. 6.4, 6.5). Root hair (small arrows) elongation has been inhibited and the number of epidermal cells (e) between adjacent root hairs is less than in control roots (see below). Fig. 6.3, X35; Fig. 6.4, X100; Fig. 6.5, X200.

Fig. 6.6 A basal portion of an untreated root approximately 2mm in length, cleared and observed using Nomarski interference-contrast optics. (e) - epidermis. X80.

Fig. 6.7 Young roots (arrows) grown continuously in colchicine solution have not elongated but their width is about twice that of control roots of the same length. Approx. X70.

Fig. 6.8 A 2mm long root grown in continuous colchicine solution, cleared and viewed using Nomarski interference-contrast optics. Root hair elongation (small arrows) has been inhibited. X100.

Figs. 6.9-6.12 Roots grown in colchicine solution for 24 hours and then in fresh nutrient medium for 3 days. The regular banded pattern of secondary wall thickenings in the xylem is completely lost, and masses of secondary wall are deposited all over the three faces of the cells. Increase in girth of the roots is usually due to lateral swelling of cortical and surface layers but the xylem cells in the root in Fig. 6.12 have also swollen laterally. Stelar cells with abnormally thick walls (arrows) were also occasionally observed, as were discontinuities in the files of xylem cells (double-ended arrows). All X320.

Fig. 6.13 10 hour colchicine treatment followed by 36 hours recovery. The ability to focus at different levels in the root using Nomarski interference-contrast optics, enabled the observation that the secondary wall material in the acrosopic xylem element (arrow), although smeared, had been mainly deposited near the edges of the cell. Only a few masses of secondary wall material occurred on the cell faces. X960.

Fig. 6.14 A xylem element in a root fixed immediately after a 24 hour colchicine treatment. Masses of electron-dense material overlie the rather "smeared" secondary wall thickenings. X30,000.
FIG. 6.15  The effects of continuous colchicine treatment on root development.

A. Daily measurement of the lengths of adjacent roots along a frond growing continuously in colchicine solution.

B. The number of merophytes in roots of plants growing continuously in colchicine for 1 day (○); 2 days (▲); and 3 days (□). The solid line represents values in control roots (see Fig. 2.49).

C. The width (as measured by the outer diameter of the root cap) of roots treated with colchicine: continuous treatment for 3 days (○); 4 and 5 days (○); 6 and 7 days (▲) and a 2 hour pulse followed by an 8 day recovery (■). The solid line represents the widths of untreated roots (see Fig. 2.48).

D. The distance from the root apex (measured by the number of merophytes) of the most acroscopic xylem element showing evidence of secondary wall deposition after continuous colchicine treatment for 1 day (○); 2 days (○) and 3 days (□). The solid line represents values in control roots (see Fig. 4.16).
FIG. 6.16 The effects of a 2 hour pulse of colchicine on root development.

A. Daily measurement of the lengths of adjacent roots along a frond growing in fresh nutrient medium after a 2 hour colchicine treatment.

B. The number of merophytes in roots of plants growing in nutrient medium after a 2 hour colchicine treatment. Recovery times: 12 hours (◊); 24 hours (△); and 36 hours (□). The solid line represents values in control roots (see Fig. 2.49).

C. The distance from the root apex (measured by the number of merophytes) of the most acroscopic xylem element showing evidence of secondary wall deposition in roots recovering from a 2 hour colchicine pulse. Recovery times: 12 hours (◊) and 36 hours (□). The solid line represents values in control roots (see Fig. 4.16).
A

Root length [mm]

0 5 10 15 20 25 30

Time [days]

1 2 3 4 5 6

B

Number of merophytes in root

0 5 10 15 20 25 30 35 40 45 50

Root length [mm]

02 04 06 08 10 12 14 16 18 20

C

Number of merophytes from apical cell

0 5 10 15 20 25 30 35 40

Root length [mm]

02 04 06 08 10 12 14 16 18 20
Figs. 6.17-6.22  The effect of a 2 hour colchicine pulse on xylem differentiation.

Figs. 6.17, 6.18  Two hour colchicine pulse followed by a 48 hour recovery period. Root cleared and observed using Nomarski interference-contrast optics. The files of xylem cells are shown at a different plane of focus at the higher magnification (Fig. 6.18). No recovery of regular wall thickening formation has occurred. Fig. 6.17, X240; Fig. 6.18, X900.

Figs. 6.19-6.20  Four adjacent xylem elements along a root fixed after a 24 hour recovery following a 2 hour colchicine pulse. The ultrastructural appearance of each cell can be compared to the arbitrary stages of development diagrammatically represented in Fig. 4.40D. The cell in Fig. 6.19 approximates to stage 4; those in Figs. 6.20 and 6.21, to stage 5; and that in Fig. 6.21, to stage 6. No effects of the colchicine on the ultrastructure of the xylem cells other than the irregularity of secondary wall deposition is evident. The apex of the root in which these cells occurred, lies to the right of the page. Fig. 6.19, X5,400; Fig. 6.20, X4,400; Fig. 6.21, X4,000; Fig. 6.22, X3,000.
Figs. 6.23-6.29  The recovery of xylem differentiation after a 2 hour colchicine pulse

Figs. 6.23-6.29  Cleared roots viewed using Nomarski interference-contrast optics. The roots have been given different colchicine pulse treatments and allowed to recover. Figs. 6.23, 6.24: 7 hour colchicine and 48 hour recovery; Fig. 6.25: 5 hour colchicine and 3 days recovery; Fig. 6.26: 2 hours colchicine and 48 hours recovery. In all roots, regular thickening formation (brackets) has resumed in xylem elements acroscopic to those in which abnormal wall depositions had occurred. Root elongation during the 3 days recovery in the root in Fig. 6.25 has stretched the abnormal secondary wall masses. Fig. 6.23, X200; Figs. 6.24, 6.25, X540; Fig. 6.26, X630.

Figs. 6.27,6.28  Xylem elements in roots fixed after 2 hours colchicine followed by 24 hours recovery. Clusters of vesicles and amorphous material overlie the irregular wall thickenings. A few microtubules are evident in the cell in Fig. 6.27 (arrows). Fig. 6.27, X22,000; Fig. 6.28, X48,000.

Fig. 6.29  A portion of a xylem element in a longitudinal section of a root fixed after a 2 hour colchicine pulse followed by 36 hours recovery. The section cuts the cell near an outer corner of the triangular-shaped cell. Bands of transversely aligned microtubules are associated with clusters of vesicles and matrix material along the edge of the cell. Serial sections showed that the vesicles and matrix material were confined to the region near the cell edge and did not extend onto the cell faces. X50,000.
FIGS. 6.30-6.35  The ultrastructure of roots recovering from a 2 hour colchicine pulse (1)

FIG. 6.30 and insert. Longitudinal sections of a 0.25mm long root fixed 12 hours after the 2 hour pulse. The apical cell contains 4 nuclear profiles (n). X4,500. Insert, X300.

FIG. 6.31  A longitudinal section through the apical cell of a root 0.2mm in length fixed 24 hours after the 2 hour colchicine treatment. Five nuclear profiles (n) are evident in the apical cell. The nucleus in the first merophyte has been arrested at metaphase (MA). X4,300.

FIGS. 6.32-6.35  Longitudinal sections through roots 0.17mm (Fig. 6.32) and 0.32mm in length (Figs. 6.33-6.35) fixed 36 hours after the 2 hour colchicine pulse. Wall formation (small arrows) has begun to subdivide the nuclei (n) in the apical cells and in the cells in the merophytes. The merophyte at the top of Fig. 6.35 lies adjacent to the apical cell. The wall dividing the 2 nuclei in this first merophyte, contains electron-dense material and has apparently been completed during the recovery period. The second merophyte along the same sector of the root, contains 3 nuclei but no new wall formation is evident. Stretches of abnormally contoured wall material in the inner cell in the third merophyte along this sector, have been cut in grazing section and in cross-section. At higher magnification (Fig. 6.34) bundles of microtubules can be seen near the wall (long arrows). The walls also possess unusual clusters of plasmodesmata (short arrows). Fig. 6.32, X3,500; Fig. 6.33, X6,000; Fig. 6.34, X11,000; Fig. 6.35, X5,000.
FIGS. 6.36-6.41 The ultrastructure of roots recovering from a 2 hour colchicine pulse (2)

FIGS. 6.36, 6.37 A cell in the formative division zone (Fig. 6.36) and a pair of outer cortex cells (Fig. 6.37) which have entered the second round of proliferative division, have been arrested at metaphase (MA). The root (0.25mm in length) in Fig. 6.36 was fixed 12 hours after treatment. The root in Fig. 6.37 was 0.2mm in length and was fixed 24 hours after treatment. No new wall formation is evident in either root. Fig. 6.36, X7,000; Fig. 6.37, X2,000.

FIGS. 6.38, 6.39 Grazing sections of longitudinal cell walls. After 24 hours recovery, microtubules are still absent from the cortical cytoplasm in a 0.2mm root (Fig. 6.38). After 36 hours recovery transversely oriented microtubules are again seen adjacent to the longitudinal walls in a 0.32mm root (Fig. 6.39). Both X22,000.

FIG. 6.40, 6.41 Longitudinal sections of a 0.32mm root fixed 36 hours after treatment. The formation of a cell plate (long arrow) for a longitudinal, formative division has not been completed, presumably due to the loss of the phragmoplast microtubules following colchicine treatment. Other cells have begun to deposit new wall material (short arrows) to subdivide the nuclei. The new walls in the dermatogen cells (Fig. 6.41) join the basiscopic parent walls at the correct location for the asymmetric root hair division (at downward-pointing arrows), although the wall has fused abnormally with the acroscopic parent wall in the top cell in the figure (white arrow). Fig. 6.40, X4,000; Fig. 6.41, X5,000.
Figs. 6.42-6.46 The ultrastructure of cells recovering from a 2 hour colchicine pulse (3)

Fig. 6.42 The top of the figure points towards the apex of the root. The root was 0.32 mm in length and was fixed 48 hours after colchicine treatment. Wall formation (arrows) has begun to subdivide the cells in the 2 merophytes in this longitudinal section. However, instead of the normal longitudinal formative division yielding a dermogen and an outer cortex cell, a transverse wall is being formed in the young merophyte. X2,200.

Fig. 6.43 During a 36 hour recovery period, wall formation completely subdivided the apical cell of this root 0.47 mm in length. X5,000.

Fig. 6.44 A longitudinal section of a root 0.57 mm in length fixed 48 hours after treatment. The root apex is at the top of the page. Examples of complete and incomplete new walls are indicated (arrows). Transverse walls have been completed in 2 of the 3 inner cortex - endodermis precursor cells in the file in the left-hand side of the root (double-ended arrows) instead of the normal longitudinal division which completes the formative division sequence for the inner cortex (ic) and endodermis (e). The transverse wall in the precursor cell in the third merophyte displays the slight basipetal displacement characteristic of the inner cortex first and second proliferative divisions. X3,000.

Fig. 6.45 A root 0.57 mm in length fixed 48 hours after treatment. New wall formation has successfully completed the asymmetric root hair division in some dermogen cells (white arrows). In other cases, although the new wall has fused at the correct location along the basiscopic wall, the placement of the wall is abnormal (black arrows). X3,300.

Fig. 6.46 An example of a complex wall mass (arrow) containing the electron-dense material which is seen in walls formed during the recovery period. X11,000.
CHAPTER 7

MICROTUBULES AND MORPHOGENESIS IN AZOLLA PINNATA ROOTS

- A CONCLUDING DISCUSSION

7.1 INTRODUCTION

The preceding chapters have described the morphogenesis of the root of the water fern, *Azolla pinnata*, with particular emphasis on the structure of the cortical microtubule arrays and the role(s) that they might play in the morphogenetic processes. The morphogenesis of this root, like that of any other multicellular plant structure, involves precise temporal and spatial control of cell division, enlargement and differentiation. Through the placement of the cell wall following cell division, and its architecture and chemical composition, the sizes and shapes of cells comprising all plant tissues are determined. In addition, modification of the wall during cell differentiation is an integral part of the development of the distinct and characteristic cell types which constitute the mature plant.

Cortical microtubules have at least a two-fold involvement in plant cell morphogenesis. The localised microtubule array which forms at pre-prophase accurately predicts the site of fusion of the future cell plate with the parent walls. This is often the first visible manifestation of the polarisation of the cell prior to cell division.
division. Once cytokinesis has been completed, the interphase cortical microtubule arrays are re-established and appear to participate in the regulation of cell shape and differentiation through their influence on the orientation of the cellulose microfibrils in the wall.

Many studies have revealed a parallelism between microfibril and microtubule orientation during primary and secondary wall formation. The capacity of the interphase microtubules to predict the form of the wall deposition has also been shown, the microtubule arrays being organised in advance of the ordered wall deposition. This prior organisation of the microtubules, both at interphase and pre-prophase, shifts one target of investigations of plant cell morphogenesis a step back in the precisely controlled sequence of events which occur during the morphogenesis of multicellular plant tissues, the crucial issue becoming one of how the cells control their cortical microtubule arrays.

Apart from general descriptions of their presence and orientation, little has become known about cortical microtubule arrays in plant cells, be they extensive or localised, at interphase or pre-prophase. Reports of their absence, following possibly inadequate studies, have contributed to uncertainty as to their requirement and function. Such reports are exemplified in the case of the pre-prophase band by the work of Cutter and Hung (1972) [see Section 5.3.2] and for interphase arrays, by Sterling (1975) [see Section 1.4.4] and Montezinos and Brown (1976). In this latter study, it is reported that in *Oocystis apiculata* the "cortical microtubules....disappear during the formation
of the (polylamellate) secondary wall"; yet, in the same organism, microtubule arrays have been described in detail during wall formation by Robinson and co-workers (Robinson *et al.*, 1976; Sachs *et al.*, 1976 [see Section 1.4.3]).

The present work was undertaken with the aim of providing a thorough investigation of the structure and occurrence of cortical microtubule arrays, with the additional hope that information concerning their organisation and function might also be revealed. It was first necessary to characterise a system on which to work, and the *Azolla pinnata* root proved to be very advantageous. It was possible to document every division which occurs during the formation and growth of the root. The pattern of pre-existing walls allowed the position of future walls to be accurately determined. Precise sequences of formative divisions yield cell initials for each cell type in the root, thus every cell could be identified. It was also possible to follow files of cells from the first (youngest) representatives at the termini of the branches of the cell lineage division system, along the root through zones of elongation, proliferative divisions, and, in some cases, differentiation. This background information is presented in Chapter 2, and will not be discussed further. Instead, the remaining discussion will concentrate upon the cortical microtubules.
7.2 The Structure and Occurrence of Cortical Microtubule Arrays.

The concept that cortical arrays consist of hoop-like microtubules has persisted ever since the original description in 1963 (see Section 3.1). This concept bestows upon the arrays a mental image of a possibly static and cumbersome structure. However, tracking of individual microtubules at interphase and pre-prophase has now revealed that the microtubules are not complete hoops but that the arrays are composed of overlapping, component microtubules, which are short relative to the dimensions of the cell. The length of the microtubules varies widely, the average length being approximately one eighth of the cell circumference. The data on the length distribution of complete microtubules shows that it is extremely unlikely that there are any microtubules whose length approaches that of the cell circumference.

The concept that emerges from this new analysis is one of a dynamic array, the form of which can be rapidly altered, locally or extensively, by additions, deletions, or changes in length. Just as the numbers and lengths of the constituent microtubules were affected experimentally by imposing physical and chemical treatments on the plants, so too could the arrays be regulated by internal and external morphogenetic forces through the availability of tubulin and initiating sites.

Interphase arrays of cortical microtubules were found throughout the root, paralleling the alignment of
the wall microfibrils during primary and secondary wall formation. The density of microtubules in these arrays was found to decrease after the final longitudinal formative division for each cell type, reaching a plateau level at which interpolation of microtubules in the array maintains a constant density of microtubules in the face of extensive cell elongation. Pre-prophase bands of microtubules were found to precede every category of division in the root, accurately predicting the site of fusion of the cell plate with the parental walls. The size of the pre-prophase band varies according to the stage of development of the band and the size and type of cell.

7.3 **The Function of Cortical Microtubules in Wall Formation**

More extensive cross-bridging than was previously envisaged has been revealed in the study of the geometry of the cortical microtubule arrays in *Azolla pinnata* root tip cells. Both the sequences of serial sections showing transverse views of microtubules, and sections showing longitudinal profiles, revealed the existence of periodic bridging between adjacent microtubules and between microtubules and the plasma membrane. The bridges between the microtubules and the plasma membrane have been postulated to be involved in achieving the orientation of the cellulose microfibrils in the wall, through the movement of the cellulose-synthesising enzymes in the membrane (Heath, 1974a; Hepler and Palevitz, 1974) [see Section 1.6.1]. Evidence
for the interaction of microtubules with the plasma membrane is not confined to plant cells (see review in Roberts, 1974). Topographical changes of the cell surface associated with loss of organised cortical microtubule arrays have been reported in a number of animal cell types (e.g. Fonte and Porter, 1974; Berlin, 1975; Edelman, 1976; Cohen, 1977).

As early as 1958 it was suggested that cellulose synthetase enzymes might be located at the growing tips of cellulose microfibrils, thereby directing attachment of glucose onto the growing chains (Roelofsen, 1958). Recent biochemical evidence indicates that in higher plants, cellulose synthesis does occur at the cell surface (Shore and MacLachlan, 1975; Shore et al., 1975; Ray et al., 1976). A single example of intracellular cellulose synthesis has been found in an alga. In Pleurochrysis, the golgi apparatus can function in the assembly of cellulosic scales and their transport to the cell surface (Brown and Romanovicz, 1976; Romanovicz and Brown, 1976; Brown and Willison, 1977).

The orientation of cellulose microfibrils in the absence of cortical microtubules appears to be confined to the Chlorophycean algae. The strict parallel alignment of microfibrils in the walls of these algae is considered to be achieved by a square-packed arrangement of cellulose synthetase-containing granules in the plasma membrane (Preston, 1964, 1974). These "ordered granules" are hypothesised to be able to synthesise and orient two sets of microfibrils lying mutually at right angles and two sets lying along the 45° axes.
In the alga, *Oocystis*, it has again been suggested that cellulose microfibril orientation is achieved by granule complexes, either in (Brown and Montezinos, 1976; Montezinos and Brown, 1976; Brown and Willison, 1977) or on (Robinson and Preston, 1972) the plasma membrane. In this case, the evidence indicates that the granule complexes possess a linear arrangement. Cortical microtubules are present during wall formation (see Section 7.1) and although granule bands associated with the plasma membrane apparently direct the deposition of parallel microfibrils, it would seem that the microtubules are required to achieve the change in the orientation of the alternate lamellae, presumably by changing the orientation of the granule bands themselves. Although not reported by the authors, cross-bridges between the microtubules and the plasma membrane are evident in the micrographs in their publication (Sachs et al., 1976).

The finding that treatment of tissues with glycerol prior to freezing could result in the modifications of the plasma membrane (Willison, 1975, 1976) has been followed by a number of observations in higher plants of particle arrays associated with the plasma membrane which do correlate with the orientation of the cellulose microfibrils in the wall (see Section 1.6.1).

If the views of shadowed preparations of the walls of the Chlorophycean algae, *Valonia*, *Chaetomorpha* and *Cladophora* (see Preston, 1974) are compared with recently published micrographs of the inner surface of the walls of cotton fibres (Willison and Brown, 1977), a marked difference
in the appearance of the wall lamellae is evident. In the algae, the microfibrils are rigidly parallel and straight but in the cotton fibre the microfibrils show a more flexible arrangement. Although, overall, they run parallel with the long axis of the fibre, the microfibrils are often in bundles, within which the microfibrils form in-phase waves of measurable wavelength and amplitude.

If the replicas of the cotton fibre wall are compared to the many maps of cortical microtubule arrays included in this thesis, similar patterns of undulations of the cortical microtubules can be seen. In the cotton fibre wall, individual microfibrils may leave one bundle at an angle and join an adjacent bundle just as individual microtubules may leave one group and slant across to another. Granules associated with the plasma membrane, and cortical microtubules lying parallel to the microfibrils in the wall, were also seen in the developing cotton fibres.

These observations give support to the hypothesis that microfibril alignment in higher plant cells is achieved by the interaction of cortical microtubules with the cellulose synthesising enzymes in the plasma membrane, possibly via the cross-bridges between the microtubules and the membrane. It is difficult to envisage how the undulations of the microfibril bundles, or the passage of individual microfibrils from one bundle to the next, could occur if the microfibril alignment was achieved through a widespread oriented flow in the membrane.

There is growing evidence that the microtubule cross-bridges participate in motility, requiring energy in
the form of ATP (McIntosh, 1973; Gibbons, 1977; Sale and Satir, 1977). Assuming a similar capacity for the cross-bridges connected to cortical microtubules, Green (personal communication) has suggested that a simple mechanism involving the maximisation of overlaps between the adjacent microtubules, which were also anchored to the plasma membrane, would be sufficient to maintain the cortical microtubule arrays in a transverse orientation in the face of sometimes extreme longitudinal cell elongation.

The analysis of the structure of the cortical microtubule arrays in *Azolla* roots has revealed a dynamic system of short, overlapping microtubules. The control of cellulose microfibril orientation by these cortical microtubule arrays offers a much more versatile system than the square-packed granule complexes which occur in the unicellular Chlorophycean algae - a versatility which may be related to the complexity of, and necessary for, co-ordinated growth in multicellular plant tissues. Cortical microtubule arrays might be regulated by the interplay of internal and external morphogenetic signals, the orientation of the microfibrils being deposited in the wall being changed accordingly. This is well-illustrated by the hormonal effects on microtubule and microfibril orientation observed in bean and lettuce hypocotyls (Section 1.4.3). The situation in the alga *Oocystis* may be an intermediate stage in the development of the control of microfibril orientation by the cortical microtubules.

The hypothesis that cross-bridges between adjacent microtubules participate in maximising the overlapping of the
component microtubules of cortical arrays, thereby maintaining transverse alignment, and the hypothesis that cross-bridges between microtubules and enzyme complexes in the plasma membrane achieve the alignment of the cellulose microfibrils, is supported by a previously inadequately explained observation made 5 years ago.

In concluding a detailed study of developing xylem elements in cucumber hypocotyls, Goosen-de Roo (1973c) examined the effects of plasmolysis on the cell protoplast. In non-plasmolysed cells, as in all other cases of xylem differentiation, bands of microtubules were seen to overlie the developing thickenings and in the plasmolysed cells, the bands of microtubules were still present at the original positions. The surface of the protoplast did not become smooth after plasmolysis but showed low, somewhat irregular, ridges at positions which were formerly located between the thickenings. It was suggested by the author that these free ridges could be explained in three ways:

1. The relief on the outer surface of the protoplast is merely an imprint of the cell wall thickening.
2. The ridges are caused by differential shrinkage of the protoplast.
3. The ridges are caused by longitudinal shrinkage of the microtubules, resulting in localised constrictions of the protoplast.

Unless held in place by the cell wall, protoplasts...
generally assume a spherical shape and hence the simple 
retention of the imprint of the wall profile is highly 
unlikely. Differential shrinkage was thought possible if 
areas of the protoplast with high densities of organelles, 
such as between the thickenings, contain less water than 
areas with low densities. On plasmolysis, areas with high 
water content would lose more water, more rapidly, resulting 
in differential shrinkage of the protoplast. This too, 
seems extremely unlikely. However, a modification of the 
third proposal can adequately explain the results. If the 
 microtubules shrink, the ridges would be formed by the 
 protoplasmic areas bulging between the constrictions. The 
author concluded that it was likely that microtubule 
 shrinkage did occur as there were "no indications that 
 the microtubules have maintained their original length in 
 the plasmolysed protoplast. If they had, one would expect 
 to find turns and loops in the microtubules. Since we 
 found only cross sections of microtubules, it is likely that 
 the microtubules did indeed shrink".

There is, however, no biochemical evidence that 
individual microtubules are able to "shrink". The obser- 
vations can be explained if the microtubule cross-bridges 
have the capacities proposed above. Normally, the plasma 
membrane is held firmly against the cell wall by the turgor 
pressure of the cell and also possibly by connections of 
growing cellulose microfibrils with enzyme complexes in the 
membrane. When the cell is plasmolysed the membrane 
shrinks away from the wall, breaking these connections. 
A tendency toward the maximisation of overlapping of
cortical microtubules which are still anchored to the membrane would then result in constriction of the protoplast in the areas occupied by the microtubule bands. In most cells, microtubules are evenly distributed along the walls and so constriction of the cortical microtubule arrays would not lead to the pattern of ridges shown by developing xylem. In all situations, however, it would be of interest to confirm the relative movement of the overlapping component microtubules by obtaining microtubule counts. An increase in frequency should accompany a decrease in cell surface area.

7.4 THE FORMATION OF CORTICAL MICROTUBULE ARRAYS

The hypothesis that cortical arrays of microtubules arise through the activities of zones along the edges of cells is discussed in detail elsewhere (Gunning, Hardham and Hughes, 1978b) but it is relevant to show how the present results conform with and, indeed, are basic to, the hypothesis.

The interpolation of microtubules throughout the interphase arrays during cell elongation implies that a capacity to initiate microtubule assembly must exist along the length of the cell. The tracking of microtubules by serial sectioning along the cell faces revealed no strong evidence for the presence of MTOCs. No structures indicative of MTOC activity were observed either throughout the arrays or associated with the occasional clusters and lines of terminations which, as pointed out in the Discussion in
Chapter 3, need not have originated where they were observed. Since the cell faces contained no MTOCs it was possible that these sites might be confined to the cell corners. The specialisation of cell corners was also suggested by the study of xylem differentiation. Thickening formation was preceded by localised clustering of cortical microtubules and was initiated in the corners of the cell.

Evidence concerning the origin and development of microtubule arrays was sought by searching short sequences of serial sections, and it was found that configurations indicative of microtubule initiation were present along cell edges, provided that the cells were examined very soon after cytokinesis, when they were setting up their interphase arrays anew (Gunning, Hardham and Hughes, 1978b).

That the microtubules are, on average, approximately one eighth of the cell circumference in length, is in accord with the concept of initiation of the arrays along the, usually four, edges of the cell (Fig. 7.1A) and the subsequent interaction of the microtubules along the cell face. The possibility that the arrays are dynamic has already been emphasised and it seems reasonable to suggest that the short microtubules encompassed in the sequences of serial sections might migrate onto the faces of the cell from the corner regions. Having detached from the MTOCs, the short lengths may be moved by mechanochemical interactions mediated by cross-bridges (Fig. 7.1B).

The observations presented in Chapter 5 on pre-prophase bands provide further background to this hypothesis. Prior to every category of division in the root, pre-prophase
bands were observed in the cell occupying a zone approximately 1.5-3.0\textmu m in width. In all cases, this zone was subsequently bisected by the cell plate when it fused with the parent walls. It has been proposed (Gunning, Hardham and Hughes, 1978b) that this specialised zone, of which each daughter cell inherits half, may be activated after cytokinesis to participate in the formation of the microtubule arrays.

The great majority of cell faces are circumscribed by edges which originate by bisection of pre-prophase band zones. Hence, it is possible that the ability to form pre-prophase band microtubules in these zones is altered, after cytokinesis, to an ability to act as the MTOC for the cell face. The vectorial properties of these postulated MTOCs remain to be investigated, but it is relevant that the positioning of C-shaped terminations (Section 3.3.1) is indicative of shared directionality of growth of the arrays. The possible role of cross-bridges in establishing and maintaining order in an initially un-ordered array of microtubules has already been discussed (Section 7.4).

More extensive evidence for the initiation of cortical microtubule arrays along the edges of cells might be obtained if the proportion of cells that are establishing their microtubule arrays could be increased. Indeed, the preliminary attempts to procure this situation using a colchicine pulse treatment did provide some supportive evidence for the hypothesis (Chapter 6), although this method of achieving microtubule depolymerisation proved to be unsuitable due to the ill-defined pulse time and the delayed and only
partial recovery. The experiments did, however, show that loss and recovery of the microtubule arrays could be effectively monitored by observation of xylem thickening formation in cleared roots in the light microscope. The data on low temperature and high pressure effects have revealed the ability of these treatments to depolymerise microtubules and possibly to allow a rapid recovery after treatment. The *Azolla* root thus may provide a system in which an optimum treatment and recovery regime can be determined and in which to investigate the re-establishment of cortical microtubule arrays in cells that can be categorised in terms of their developmental history and stage of differentiation.
FIG. 7.1 The initiation and organisation of cortical microtubule arrays in plant cells.

A. Following cell division, re-establishment of the cortical microtubule arrays along the longitudinal walls of the cell occurs through the activity of microtubule organising regions situated along the edges of the cell. At early stages of microtubule initiation and assembly, vesicles and dark-staining matrix material are associated with the microtubules near the cell edges.

B. Interaction of the microtubules via the cross-bridges distributes short lengths of microtubules throughout the array. Maximisation of overlaps between microtubules establishes and maintains the transverse alignment of the mature array.
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