CELL RESPONSE TO TISSUE WOUNDING IN
STEMS OF Datura stramonium L.

by

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Frontispiece: Figure 1.1:

A pith cell from a stem 4 days after wounding that has been isolated by extraction with EGTA and opened-out by hand dissection. As seen as a sheared (double) image in white light using uniform field interference optics. Differences in optical thickness are apparent as a difference in colour within the specimen.

The two end walls lie to the left of the side wall, which has been completely unfolded. All walls are a single thickness only. Longitudinal bands of different (greater) optical thickness can be seen running the length of the side wall, but not encroaching upon the end walls. These thickenings are located at the site of intercellular spaces and are separated by pitted areas of wall face. The bands remain visible despite the considerable deposition which takes place on all the walls of the cell during the pre-mitotic wound-response. Further details are given in Chapter 3 and 4. A black and white image of the same specimen, as seen in monochromatic light, is shown in Figure 4.17b.
STATEMENT

All the work presented in this thesis, except where due acknowledgement is made, is the author's and has not been previously submitted for any other degree.

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have ever finished.
ABSTRACT

This thesis describes the early cytoplasmic and cell wall changes that occur when stem pith cells of *Datura stramonium* L. are induced into a novel direction of expansion and re-differentiation by tissue wounding. Removal of a portion of the vascular cylinder stimulates the previously quiescent pith cells to re-enter the mitotic cycle and eventually to replace the lost tissue. The vast majority of the initial divisions are parallel with the wound and the resultant tissue-expansion is changed from longitudinal in unwounded stems through 90° to be normal to the long axis of the stem.

The cytoplasmic response was studied, both by use of light, scanning-electron and immunofluorescence microscopy of hand sections and by light and electron microscopy of embedded material. Cell wall architecture, before and after wounding, was documented by using polarising and interference microscopy, supplemented by electron microscopy, to examine hand-carved individual walls and cut-open whole cells.

Prior to wounding, the cells are large and highly vacuolated, with only a thin layer of cytoplasm. The cell walls conform to classical descriptions, with predominantly transversely-oriented cellulose on the side (longitudinal) walls. This orientation continues onto the oblique end walls, whereas transverse end walls have randomly-oriented microfibrils. In all cases cortical microtubules and recently-deposited wall microfibrils are congruent. The side walls possess longitudinal ribs of wall thickening, consisting of external longitudinal microfibrils, against intercellular spaces or at their margins. Internally, extra transverse microfibrils and additional matrix materials are found at these sites.

After wounding, considerable cytoplasmic reorganisation occurs. The nucleus becomes enclustered by organelles and undergoes a traumatotactic
migration. A systrophe, or cytoplasmic star, containing the nucleus, then forms, which thickens to form a phragmosome in which mitosis and cell plate formation occur. Phragmosomes and some systrophic arms predict the line of the new cell-wall and often abut one another in adjacent cells. Pre-prophase bands also form during the systrophic stage, similarly predicting the plane of division. The initial division occurs 3 or 4 days after wounding, after which subsequent cell-cycling takes place rapidly and a marked radial expansion occurs in the responding tissue. Prior to the first round of division there is a massive laying-down of cellulose microfibrils in a polylamellate fashion, with an overall longitudinal bias, against all walls of the cells. This deposition presumably allows for the novel direction of expansion that occurs upon resumption of meristematic activity. The time course of deposition was monitored semi-quantitatively by polarising and interference microscopy. The novel walls are shown to be deposited in an orthogonal fashion, with microtubules playing the major role in determining the direction of deposition. The data do not support the concept of polylamellate walls arising through 'self-assembly' in a helicoidal manner.

The roles of microtubular 'hoops' in cell wall deposition and of systrophes, phragmosomes and pre-prophase bands in determining the plane of division are discussed.
LIST OF ABBREVIATIONS USED THROUGHOUT THIS THESIS

(a) Abbreviations used in the text:

BSA bovine serum albumin
\( \Gamma \) retardation
A-DIC amplitude differential interference contrast
d optical thickness
DIC differential interference contrast
EGTA ethylene glycol-bis (\( \beta \)-amino-ethyl-ether)
\(-N,N'\)-tetra-acetic acid
EM electron microscopy
ER endoplasmic reticulum
F-actin filamentous actin
FITC fluorescein
GAM 20 goat anti-mouse IgG coupled to 20nm colloidal gold
GMA glycol methacrylate
Ig immunoglobulin
IgG immunoglobulin G
MAB monoclonal antibody
MAP microtubule-associated protein
MSB microtubule-stabilising buffer
MTOC microtubule-organising centre
n refractive index
NBD-Ph NBD-phallacidin (7-nitrobenz-2-oxa-1,3-diazolylphallacidin)
OPD optical path difference
PBS phosphate-buffered saline
Pd phalloidin
PEG 200 polyethylene glycol 200
PEG 400 polyethylene glycol 400
PLL poly-L-lysine
PMSF phenylmethysulphonyl fluoride
PPB pre-prophase band
PS perfusion solution
RAM-FITC fluorescein-conjugated rabbit anti-mouse IgG
RAM-TRITC rhodamine-conjugated rabbit anti-mouse IgG
RT room temperature
SEM scanning electron microscopy
TRITC rhodamine
T-X Triton-X 100
UV ultraviolet
(b) Additional abbreviations used in the Figure Legends:

A  amyloplast
Co  collenchyma
D  dictyosome
E  end wall
Ep  epidermis
ER  endoplasmic reticulum
I  intercellular space
IP  inner periclinal wall
M  mitochondrion
N  nucleus
O  obliquely-oriented end wall
Oc  outer cortex
OP  outer periclinal wall
P  pit
Ph  phloem
Pi  pith
R  radial wall
W  wound
Xy  xylem
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INTRODUCTION

This study reports the investigation of the enzymatic and catalytic changes caused during the early stages of the response of yeast to certain physiologically active substances. This chapter will describe the studies related and the background physiology.
This thesis reports the investigation of the cytoplasmic and cell wall changes that occur during the early stages of the response of stem pith cells of a dicotyledonous species to tissue wounding. This chapter will describe the species involved and the background plant development.

1.1 DATURA STRAMONIUM L. - AN OVERVIEW

The plant used throughout this study is Datura stramonium L.. Datura is one of several narcotic genera in the Solanaceae, others include Nicotiana (tobacco), Atropa (belladonna) and Mandragora (mandrake). The generic name Datura was first used by Linnaeus in his Hortus Cliffortianus of 1737, the term coming either from the Hindi 'dhatura' or 'dutra' or from the Arabic 'tatorah' (references in Satina and Avery, 1959). It is a genus of ancient and world-wide interest. In India, the drug obtained from Datura was used by thugs to stupefy their victims and as a treatment for rabies. In South America, extracts were used in shamanistic ceremonies and to deaden the senses of those who were to be buried alive. The Aztecs, who regarded the seeds as sacred, used Datura as a treatment for many diseases and as an intoxicant to facilitate communication with spirits. The Indians of southern North America also used Datura both therapeutically and ritually (the preceding and other historical aspects are given by Avery, 1959). More recently, the alkaloids that Datura contains have been used for a number of medicinal and drug purposes (Leete, 1959).

The origin of the specific name, stramonium is moot. The Oxford English Dictionary cites its probable etymon as the Tartar 'turman' meaning horse-medicine. On the other hand, Asa Gray (cited by Satina and Avery, loc. cit.) stated that the name came from the modified Latin, struma or strama, meaning a swelling. The common-names for D. stramonium usually
refer to its spiny, capsular fruit or to its toxic properties. Its general name in English-speaking countries is 'thorn-apple' but other names are '(false) castor oil plant' in Australia, 'stramny', 'raving night-shade' or 'angel's trumpet' in the U.K., 'pomme épineuse' or 'herbe aux sorciers' in France, 'yosho' ('foreign') in Japan, and 'stinkweed' or 'Jimson weed' in the U.S.A. (Maiden, 1920; Parsons, 1973; Satina and Avery, loc. cit.). The last of these names is a corruption of Jamestown, Virginia, where the dramatic effect of *D. stramonium* on the British soldiers sent there to quell Bacon's Rebellion in 1676 is vividly described by Beverley in his 'History of Virginia' (in Avery, and Maiden, loc. cit.):

'The James-Town Weed, which resembles the Thorny Apple of Peru,...is supposed to be one of the greatest Coolers in the World. This being an early Plant, was gathered very young for a boiled salad, by some of the soldiers...and some of them ate plentifully of it, the Effect of which was a very pleasant Comedy; for they turn'd natural Fools upon it for several days: One would blow up a Feather in the Air; another would dart Straws at it with much Fury; and another stark naked was sitting up in a Corner, like a Monkey, grinning and making Mows at them; a Fourth would fondly kiss, and paw his Companions, and sneer in their Faces, with a Countenance more antick, than any in a Dutch Droll. In this frantick Condition they were confined, lest they should in their Folly destroy themselves; though it was observed, that all their Actions were full of Innocence and good Nature. Indeed, they were not very cleanly; for they would have wallow'd in their own Excrements, if they had not been prevented. A Thousand such simple Tricks they play'd, and after Eleven Days, return'd to themselves again, not remembering anything they had pass'd.'

The more tragic effects of *D. stramonium* upon humans (and animals) in nineteenth-century Australia and elsewhere are reported by Maiden (loc. cit.).

In the middle portion of this century, the genus *Datura* received considerable attention as a genetic research tool - the first haploid vascular plant to be discovered was a *Datura* - especially by Blakeslee and his co-workers, e.g., see the eulogy to him edited by Avery et al.
(1959). Indeed, in the introduction to that volume Sinnott states that, 'Few organisms have yielded such a wealth of scientific information as has come from this plebeian weed', and, in a case of *sic transit gloria mundum*, 'No other organism, not even *Drosophila*, has involved so many aspects of biology in its genetic investigation as has *Datura*'.

Despite it no longer being a major focus of genetic research, *Datura* is still used as a tool in a number of scientific fields. These include applied studies, as *D. stramonium* is a major weed in many summer crops, particularly cotton, maize, peanuts and soybeans in southern Africa, North and South America and Australasia (Felton, 1979; Parsons, 1973). In addition to competing for moisture, light and nutrients, a single plant of *D. stramonium* may produce up to 23,000 seeds in a single season (Parsons, loc. cit.). The seeds can remain viable in the soil for over 20 years (see Monaghan and Felton, 1979). They may release tropane alkaloids which are able to exert an allelopathic effect on germinating crop plants (Levitt *et al.*, 1984). In addition to alkaloids, the lectin from *D. stramonium* has been isolated (Kilpatrick and Yeoman, 1978) and used in immunochemical and immunofluorescent studies (Jeffree and Yeoman, 1981; Jeffree *et al.*, 1982; Kilpatrick *et al.*, 1980). The early genetic work has been extended to tissue cultural studies (e.g., Engvild, 1973), especially the use of *Datura* protoplasts for regeneration (Schieder, 1975, 1976) and hybridisation (Schieder, 1977, 1978a, b). However, more pertinent to the work in this thesis is the use of *D. stramonium* in grafting experiments (e.g., Warren Wilson, 1982; Warren Wilson and Warren Wilson, 1963, 1981, 1983; Yeoman and Brown, 1976; Yeoman *et al.*, 1978) and, especially, in studies of tissue response to wounding (e.g., Evans, 1979; Warren Wilson, 1978; Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1961a, b, 1983; Warren Wilson *et al.*, 1983).
1.2 GENERAL INTRODUCTION

The distinguishing features of different plant tissues and organs stem from the characteristics of their constituent cells. The size, shape and function of these cells are determined by the plant through control of the timing and direction of cell division and cell expansion as well as the mode of cell differentiation (e.g., see articles in Barlow and Carr, 1983; Lloyd, 1982). It is apparent, therefore, that the study of the fundamental mechanisms involved in the control of these processes is of prime importance in the understanding of the cellular basis of morphogenesis in plants.

Many studies demonstrate that elements of the cytoskeleton, especially microtubules, are major participants in plant morphogenesis. The behaviour of the cytoskeleton is well documented for a wide range of cell divisions and differentiation events that occur in the normal course of plant development. However, understanding of the underlying mechanisms remains elusive, and it was considered at the commencement of the project described in this thesis that new insights might be gained by analysing the role of the cytoskeleton in perturbed development - such as the 'unplanned', but, nevertheless, highly organised polarity* shift and divisions that often follow tissue wounding. Some aspects of these events have already been examined in a few cases, and are described in more detail in subsequent chapters (e.g., Hardham and McCully, 1982a, b; Hardham et al., 1980; Selker and Green, 1984). The work reported here focussed on the roles of the cytoskeleton in the resumption of meristematic activity in previously quiescent, differentiated cells responding to tissue wounding. The study relates especially to the role of microtubules in cytokinesis, cell-shaping

*The term 'polarity' is used throughout this thesis in the sense of the past, present or prospective main axis of growth of a cell or tissue (see Green and Lang, 1981; Selker and Green, 1984).
and cell polarity in normal plant development and so a résumé of these topics follows.

Plants have to insert their new walls in specified locations because subsequent spatial adjustments can only be slight. The determination of the 'division site' (Gunning, 1982a) where the cell plate will fuse with the parental wall is therefore of prime importance in plant development. The division site is usually selected prior to the onset of mitosis. One of the main predictors of organised divisions in plant cells is the pre-prophase band (PPB) of microtubules. This is a specialised array, first described by Pickett-Heaps and Northcote (1966a, b), which is generally 2-3 µm wide and cinctures the cell cortex at, with only one exception (see below), the position that the periphery of the new cell wall will later occupy. In fixed material, the microtubules of the PPB, overlap, are from one to several µm in length and are cross-bridged to one another (Hardham and Gunning, 1978). The total number of microtubules in any cross-section varies from 10 to over 100, in layers of one to several deep (see Gunning and Hardham, 1982). PPBs have been observed in many taxonomic groups and a great diversity of cell and tissue types. These include root and shoot meristems, cambia, leaves, uniseriate trichomes, and 2- and 3-dimensional aggregates of cells - see Gunning (1982a) for a full review. Since that article was published, papers on PPBs in new groups and cell types have included Brown and Lemmon, 1984; Galatis et al., 1982, 1983, 1984a, b. In addition to PPBs occurring in planned development, as epitomised by the Azolla root, they also precede some 'unplanned' divisions such as those occurring in response to wounding (Hardham and McCully, 1982a; Venverloo et al., 1980). Although seemingly ubiquitous in organised division in higher plants where cell-wall fusion occurs (Gunning, 1982a), PPBs are not always present in organised divisions amongst the lower plants. For example, they
are absent in apices of the alga Chara (Pickett-Heaps, 1975) (indeed, they have not yet been found in any fungus or alga), in sporophytes of the hornworts Phaeoceros and Notothylas (Brown and Lemmon, 1985), and in protonemata of the moss Funaria (Schmiedel and Schnepf, 1979a), although they are present in leafy gametophores of the latter (Schmiedel et al., 1981).

Despite the numerous descriptions of PPBs, relatively little is known about the development of the PPB and its exact mode of function. Furthermore, the determination of the division site in general, and not only of the PPB, is largely unknown. A variety of factors, such as internal genetic controls and positional information are apparently responsible (Gunning and Hardham, 1982). That a delicate balance of influences exists and that, in some cells, intercellular factors are involved has been demonstrated by the observation of two PPBs (Galatis et al., 1983) and the misplacement of the cell plate relative to the PPB (Galatis et al., 1984b) in 'double polarised' cells. However, such external influences cannot be the sole determining factor as PPBs occur in at least some uniseriate hairs (Busby and Gunning, 1980).

Evidence concerning the formation of PPBs is of two contrasting types. In Azolla roots, the band appears to form from nucleating sites along the cell edges (Gunning, 1980, 1981; Gunning et al., 1978c). However, no such microtubule-organising centres (MTOCs) were observed in the electron microscopic studies of Sphagnum leaflets (Schnepf, 1984) or in immunofluorescence investigations of onion root tips (Wick and Duniec, 1983). In the latter study it was deduced that the PPB gradually narrowed from an initially wide band of microtubules only slightly more dense than the surrounding cortical arrays. As the authors pointed out, the explanation of PPB formation must be more complex in cells where the
orientation of the PPB differs from the orientation of the interphase microtubules (e.g., Busby and Gunning, 1980; Galatis and Mitrakos, 1979; Gunning et al., 1978b).

There have been several proposals as to the function of PPBs. Initially, such ideas focussed on the function of the microtubules themselves, but it seems likely that the prime role of PPBs is in the establishment of the division site in the cell cortex (see Gunning, 1982a). It has been suggested that the microtubules of the PPB are precursors of spindle microtubules (see Pickett-Heaps, 1974) or are involved in the deposition of extra wall material at the site of the band (Galatis and Mitrakos, 1979; Packard and Stack, 1976). It has also been proposed that the PPB may position and orient the nucleus before mitosis (Burgess, 1970; Burgess and Northcote, 1967). However, whilst PPB formation often precedes nuclear migration to the same plane, for example in some of the divisions in the Azolla root (Gunning et al., 1978b), the order is reversed in other areas, such as in subsidiary cell formation in Commelina (Pickett-Heaps, 1969a). A 'master' influence would thus seem to be involved.

As to the site of the PPB, there is evidence arising from the work on Azolla (Gunning et al., 1978c) that the zone bisected by the new cell plate acts as an MTOC after cytokinensis for the development of interphase cortical arrays in the daughter cells (Gunning, 1980; Hepler and Palevitz, 1974). However, this does not appear to be the case either in onion root tips studied by immunofluorescence (Wick et al., 1981) or in Sphagnum leaflets studied by electron microscopy (EM), where interphase microtubules appeared to originate in the cytoplasm adjacent to the central part of the young cell wall, and not at its edges (Schnepf, 1984). It has recently been proposed that a major function of the PPB, or the site that it
previously occupied, is to prevent the cell cleavage that occurs in animal
cells (O'Brien, 1983). However, it is not immediately apparent how this
hypothesis correlates with current views on the mechanisms of cytokinesis
in animal cells. These require the assembling of a highly-ordered actin-
containing contractile ring to constrict the cells (Arnold, 1976;
Schroeder, 1972; White and Borisy, 1983) rather than the invoking of some
natural tendency of cells to cleave that has to be suppressed if it is not
to be expressed.

There is now a wealth of evidence that the *raison d'être* of PPB
formation is to generate a 'receptive site' (Gunning, 1982a) for the
margins of the growing cell plate (see Busby and Gunning, 1980; Hepler and
Palevitz, 1974). This has recently been elegantly demonstrated by Galatis
*et al.*, (1984a, b) who showed during the formation of subsidiary and hair
mother cells in *Triticum* that the cell plate was guided to the cortical
zone previously occupied by the PPB. The natural and centrifugally-induced
mis-alignments that the authors sometimes observed demonstrated that the
cell plate has to be within a certain distance before the cortical site can
exert its influence.

The PPB is not the only pre-mitotic predictor of the position of the
new cell wall that may be involved in the establishment of the division
site. Whilst observations of PPBs are a product of the era of EM, the
other early indicator, the phragmosome, was first named 25 years earlier by
Sinnott and Bloch (1940, 1941a, b) (see Chapter 2 for a further review of
the literature pertaining to phragmosomes). Phragmosomes consist of a
column of cytoplasm that contains the nucleus and in which division takes
place. They form prior to mitosis, but, thus far, have only been observed
in naturally or artificially (e.g., Ōta, 1961) vacuolated cells. Although
Sinnott and Bloch (loc. cit.) realised the importance of phragmosomes from the fact that they predicted the line of the future cell plate, they were studied relatively little until recently. The instigators of this Renaissance appear to be Venverloo and co-workers whose initial studies (Venverloo et al., 1980) raised speculation as to whether phragmosomes are also found in non-vacuolated cells and if factors which distinguish them from the surrounding cytoplasm are to be found in the bulk of the cytoplasm itself or in its cytoskeletal elements (e.g., see Gunning, 1982a; Lloyd and Barlow, 1982). Indeed it has recently been proposed that in addition to the four different microtubular populations that are generally recognised as occurring in plants, i.e., interphase arrays, PPBs, mitotic spindles and phragmoplast microtubules (Ledbetter, 1967), there is a fifth species — phragmosomal microtubules. These are reported to be aligned along the axis of the phragmosome from pre-prophase to cell plate formation inclusively and it is suggested that they play a role in the guidance of the growing margin of the cell plate to the areas of influence of the cortical site previously occupied by the PPB (Goosen-de Roo et al., 1984) in non-vacuolated as well as vacuolated cells.

In addition to there being little information concerning the exact composition and occurrence of phragmosomes (their ubiquity in vacuolated cells is even denied by some workers, e.g. Ball, 1969; Esau and Gill, 1965), very little is known about the relationship between PPBs and phragmosomes. The timing of the appearance of the two structures has only been (partially) investigated in one system (Venverloo and Pronk, 1982; Venverloo et al., 1980) and many of the studies on phragmosomes have been on tissue-cultured cells (e.g., Roberts and Northcote, 1970) where PPBs do not occur. The respective roles of the PPBs and phragmosomes in establishing the division site are clearly of importance and in need of further study.
In addition to PPBs and phragmosomes, other forms of protoplast reorganisation occur in the prelude to mitosis and in the establishment of the division site. Many of these, directly or indirectly, involve elements of the cytoskeleton. The margin of the phragmosome is anchored more strongly than other parts of the plasma membrane when the cell is subjected to plasmolysis (Sinnott and Bloch, 1941a). Similarly, Sachs (1972) showed that slow, severe plasmolysis resulted in the shrunken protoplast lying in the plane of the future division. This was so, even after a change in polarity in response to wounding. In this regard, Kirschner and Sachs (1978) noted cytoplasmic strands anticipating the plane of division. These are presumably precursors of phragmosomes. A cytoskeletal basis for preferred adhesion may be indicated by the observations of a reticulate band of cytoplasm at the edge of phragmosomes (Roberts and Northcote, 1970; Venverloo et al., 1980).

Another sign that a division site is being established is that nuclear migration occurs, as takes place in *Commelina* and *Azolla*. This has been mentioned in the foregoing, in relation to PPB formation. There are several other examples of nuclear migration, many of which involve microtubules seemingly laying a path for the nucleus (see Gunning and Hardham, 1982), such as in fern (*Onoclea*) spores (Bassel et al., 1981), and in moss protonemata (*Adiantum*; Wada et al., 1980) and caulonemata (*Funaria*; Schmiedel and Schnepf, 1980). In higher plants, nuclei and perinuclear cytoplasm often migrate during stomatal development, such as in *Vigna* (Galatis and Mitrakos, 1980). Nuclear migrations also occur in response to wounding. This is discussed further in Chapter 2.

In addition to the nucleus migrating to a division site, the position of the nucleus may also influence the spatial regulation of cytokinesis itself. This has been shown by means of centrifugation experiments,
beginning with those by Van Wisselingh (1909) on *Spirogyra* and more recently by work on *Funaria* by Schmiedel and Schnepf (1980). In both cases, the position of the nucleus at critical times influenced the position of new cross-walls. In some instances PPBs may form adjacent to displaced nuclei (see Gunning, 1982a).

After the division site has been established, mitosis occurs. The transition of microtubules from PPB to spindle has recently been described using EM (Schnepf, 1984) and immunofluorescence (Wick and Duniec, 1984). In both cases it was apparent that the 90° switch from the PPB to the spindle is not abrupt. Perinuclear microtubules initially appear aligned parallel with the PPB. Spindle formation also begins along the nuclear envelope with microtubules following the contour of the nucleus, ultimately converging upon two focal points at its surface. After the completion of spindle formation and the breakdown of the nuclear envelope, mitosis ensues - the mechanism of which is still moot (e.g., see Pickett-Heaps et al., 1982). During late telophase the phragmoplast starts to form. This comprises two sets of microtubules of opposite polarity to each other, both having their 'plus' (growing) ends at the equator (Euteneuer and McIntosh, 1980; McIntosh and Euteneuer, 1984). The spindle and phragmoplast arrays may overlap temporally as well as spatially (see Gunning, 1982a). Within the phragmoplast the cell plate develops centrifugally, with vesicles, probably of dictyosome origin and transported by the microtubules (e.g., Hepler and Jackson, 1968), fusing in a network of endoplasmic reticulum (e.g., Hepler, 1982). As the cell plate extends, the microtubules become confined to the growing perimeter, only surviving at a particular location until vesicles at that region have become part of the cell plate (e.g., Bajer, 1968).
In some cases, for example all of the divisions of the *Azolla* root primordium (e.g., Gunning, 1981, 1982b), the spindle-equator/phragmoplast machinery is in the same alignment as the PPB and the cell plate grows directly to the division site. However, in many cases, initial misalignment followed by reorientation occurs and in the case of spindles in *Allium* stomata formation, this has been shown to require energy and involve actin-like material (Palevitz, 1980; Palevitz and Hepler, 1974). In addition, there are several other examples of a strong 'pulling-force' between the growing margins of the cell plate and the division site (Galatis *et al.*, 1983, 1984a, b; Gunning, 1982a; Gunning and Wick, 1985). In Ōta's (1961) centrifugation- and Gunning and Wick's (1985) cell-piercing- experiments on dividing *Tradescantia* stamen hairs, displaced cell plates grew in a curved fashion back towards the equator of the cell, where a PPB would formerly have been located (Busby and Gunning, 1980) and hence a division site established (Gunning, 1982a). A similar phenomenon was observed by Galatis *et al.*, (1984a) in centrifuged subsidiary mother cells of *Tritium*.

The processes described above, which lead to the fusion of the cell plate with the parental walls at a predetermined site, are obviously the prime determinants of initial cell shape. However, the final shape of the cell within the organ - and therefore the polarity of the organ itself - depends upon the manner of expansion of the cells once formed. Again, microtubules appear to be a key factor in the determination of cell polarity. In this case, it is the interphase array of cortical microtubules that reforms after cytokinesis has occurred. The exact mechanism of the reinstatement (and indeed subsequent maintenance) of these arrays is not fully understood (see Gunning and Hardham, 1982). In *Azolla*, 'nucleating sites', from where microtubules appear to originate, are
observed at cell edges (Busby and Gunning, 1983; Gunning, 1980, 1981; Gunning et al., 1978c; Hardham and Gunning, 1979). In stomatal guard cell formation, MTOCs have been observed both at edges and in other cortical regions (Galatis, 1980, 1982; Galatis and Mitrakos, 1979; Galatis et al., 1983; Palevitz, 1981). However, in Sphagnum diffuse 'organising zones' (Schnepf, 1984) were observed rather than distinct foci (see also the immunofluorescence observations of Wick and co-workers described above).

The role of the cortical microtubule arrays, once established, in the initiation and maintenance of polarity is outlined in the following paragraphs.

Ledbetter and Porter (1963) were the first to observe that the cortical microtubules were generally parallel to each other; close to, and parallel with the plasma membrane and arranged circumferentially around the walls of the cell, mirroring the orientation of cellulose microfibrils in the adjacent wall. This led these authors to suggest that cortical microtubules were responsible for regulating the direction of cellulose deposition. This proposition has since received much support, both from numerous examples of microtubule/microfibril parallelism and from the effects of anti-microtubule drugs such as colchicine (see Gunning and Hardham, 1982; Lloyd, 1984a; Robinson and Quader, 1982; for recent reviews). Some of the diverse systems that illustrate cellulose/microtubule parallelism are outlined below.

The unicellular green alga Oocystis solitaria (see review by Robinson and Quader, 1982) has been used extensively in demonstrating the relationship between microtubules and microfibrils during production of its secondary wall. This is composed of lamellae criss-crossing at 90° to one another. Underlying the wall, cortical microtubules are seen cross-bridged to the plasma membrane and nearly always parallel to the innermost layer of
cellulose microfibrils (Robinson and Quader, 1980). Colchicine treatment removes the microtubules, but the microfibrils continue to be deposited in a parallel, ordered fashion although there is no switching of direction. After the washing out of the colchicine the criss-crossing of the wall layers returned (Robinson and Quader, 1981). These, and many other studies, show both that microtubules are not a pre-requisite for the production of cellulose and that they are not necessarily required for the continued growth of microfibrils in parallel arrays once parallelism has been established. Another major conclusion is that without microtubules the cell loses its ability to change the angle of directed cellulose deposition.

Secondary wall thickening in xylem cells provides another much-studied example of microtubule/microfibril parallelism (e.g., Brower and Hepler, 1976; Hardham and Gunning, 1979, 1980; Hepler and Palevitz, 1974). Microtubules become grouped into spaced, transverse clusters at sites that subsequently develop into transverse thickenings. Colchicine and other anti-microtubule drugs cause disruption of the organisation of the bands, but not their obliteration.

Studies on microtubule/microfibril parallelism are not limited to orthogonal systems. Perhaps most striking are the observations of microtubules radiating from the region of the future pore during guard cell differentiation. Prominent wall thickenings arise beneath these arrays in which the newly-deposited microfibrils parallel the microtubules. This has been observed in a number of species from mosses (Sack and Paolillo, 1983) to grasses (Galatis, 1980) to dicotyledonous species such as *Vigna* (Galatis and Mitrakos, 1980) - see Busby and Gunning's (1984) article on parallelism in *Anolla* stomata for a complete list of species studied. Strict parallelism between microtubules and microfibrils is also maintained during
the reversal of gyre that occurs within a single tier in cotton fibres (Yatsu and Jacks, 1981).

In addition to the above, there are electron microscopic observations of adjacent microtubules and microfibrils in different orientations. An early observation was in the zone immediately behind the growing tip (which has randomly-oriented cellulose) of radish root hairs (Newcomb and Bonnett, 1965), where no parallelism between microtubules and microfibrils was seen. However, a thorough re-investigation (Seagull and Heath, 1980) has shown that, in the zone of secondary wall formation behind the tip, microfibrils, when parallel, are always accompanied by similarly-aligned microtubules. In several systems where a switch in the direction of cellulose deposition occurs, microtubules may be seen to be mis-aligned when compared with the orientations of the innermost (most recent) microfibrils, such as in collenchyma (Chafe and Wardrop, 1970), and epidermal cells (Sawhney and Srivastava, 1974) - see Robinson and Quader (1982) for other examples. As initially noted by Chafe and Wardrop (1970), these observations of mis-alignments may well represent microtubules which are re-positioning prior to the deposition of microfibrils in a new direction.

On the other hand, there do appear to be structures amongst lower plants (e.g., algal scales - see Gunning and Hardham, 1982) where ordered configurations of wall microfibrils are deposited without the participation of microtubules - as opposed to those systems described above where randomly-oriented microfibrils (e.g., during tip growth) are deposited or microtubules set up an alignment which is maintained upon their removal. However, for the vast majority of plant systems, from unicellular algae to dicotyledonous species undergoing a polarity change (see below), to quote Robinson and Quader (1982), 'the weight of evidence is now so great in
favour of microtubules that it is difficult to deny them a role in microfibril orientation. Certainly there are examples which appear to be in disagreement with this, but none have been subjected to a critical examination.

Having surveyed the evidence for cortical microtubule involvement in the orientation of nascent microfibrils, two further factors will now be considered. Firstly, a brief résumé of the postulated mechanisms of microtubular control of cellulose deposition will be given. Secondly, the role of the orientation of the microfibrils, once deposited, in cell polarity and expansion will be introduced.

All the proposed mechanisms for microtubule control of cellulose deposition assume that complexes of cellulose synthetases ('terminal complexes', Mueller et al., 1976) move in the plane of the plasma membrane with their cellulose following on. In a recent review by Heath and Seagull (1982) the main hypotheses were classified according to postulated direct or indirect interaction between the microtubules and microfibrils and this convention is used in the following.

The first, an indirect, hypothesis was proposed by Hepler and Foskett (1971) and later restated by Hepler and Palevitz (1974). It is based upon the microtubules, via cross-bridges, generating an oriented flow or shear within the membrane, which aligns nearby-forming-microfibrils. In another indirect mechanism, Herth (1980) proposed that the force generated by the crystallisation of cellulose chains into microfibrils could propel the terminal complexes and that the underlying microtubules would restrict the fluidity of the membrane in such a way that the enzyme movements were channelled parallel to the microtubules. An alternative indirect hypothesis is that of Schnepf (1974), who suggested that inter-microtubule bridging and sliding contracts the array in the long axis of the
microtubule and, given fixed microtubule-plasma membrane links, causes the plasma membrane to pull away from the wall, thus creating an extracytoplasmic channel where cellulose is deposited.

The latest proposal is also an indirect hypothesis. Mueller and Brown (1982a, b) envision a flowing plasma membrane orienting the lateral movement of terminal complexes and the microtubules canalising the flow so that unidirectional deposition occurs.

The original direct hypothesis, that of Heath (1974), proposed that mobile terminal complexes move along stationary microtubules via bridging molecules such as dynein. This hypothesis was apparently incompatible with the observation that, in fixed material, microtubules are shorter than the adjacent microfibrils. For example, interphase microtubules are generally 1-4 \( \mu \)m long (e.g., Hardham and Gunning, 1978), whilst microfibrils are between 5-10 \( \mu \)m long (Preston, 1974). The hypothesis was thus modified to enable terminal complexes to 'switch tracks' from one microtubule to an adjacent overlapping one (see Heath and Seagull, 1982). However, this alteration may not be necessary as evidence is accumulating that microtubules in unfixed tissue are considerably longer than in fixed samples (e.g., Lloyd, 1984a).

A later hypothesis (Seagull and Heath, 1980), postulated that whole microtubules, with attached terminal complexes, are moved through the cell cortex by an interaction between microtubules and actin microfilaments. A similar direct mechanism was postulated by Lloyd et al., (1979b). It was suggested that terminal complexes are cross-bridged to the microtubules and the microtubules to each other. Microtubule-microtubule sliding would then move the terminal complexes along the plane of the membrane.

All of the above hypotheses involve speculation and all, in one form or another, fit the available data. Whatever the mechanism, it is clear
that some form of control exists over the deposition of cellulose. The importance of the actual direction of this deposition in relation to cell polarity and expansion is now introduced.

A typical longitudinal plant organ such as a root or a stem extends in the direction of the cell files within it. The basic driving force behind the expansion of the cells is turgor pressure; without it there is no growth (Ray et al., 1972). However, the stress in an elongate cylindrical structure, such as the majority of cells within a longitudinally-expanding plant organ, is principally transverse (Green, 1963). Therefore, the longitudinal walls of a cylinder extending along its length, characteristic of most higher plant cells except tip-growing cells such as root hairs, cannot be isotropic, but must be reinforced in the transverse (circumferential) direction. In plant cell walls, the only structures with a sufficiently high tensile strength to accomplish this are the crystalline cellulose microfibrils (Green, 1980). Hence, the longitudinal walls of most cells expanding along their long axis possess circumferential cellulosic reinforcement (Frey-Wyssling, 1959). Thus it can be seen that, once a cell has been initially shaped by the processes that determine the plane of division, its polarity (subsequent direction of expansion) depends upon the orientation of its cellulose microfibrils, which is determined, to a large extent, by the cortical microtubular array (above).

The idealised transverse reinforcement of a cylindrical cell is typified by the internodal cells of filamentous algae such as Nitella (Green, 1962). In higher plants the algal filaments may be considered analogous to longitudinal files of cells. Put simply, those in concentric circles comprise an organ with total as well as local circumferential reinforcement (Green and Brooks, 1978). Polarity of the organ is thus generated by the circumferential reinforcement of its constituent cells.
with cellulose microfibrils. As the cells expand, the layers of cellulose within the walls are thinned out and the microfibrils are displaced in the (longitudinal) direction of strain. With the continued addition of new transverse layers at the plasma membrane, a gradient of orientations from transverse to near longitudinal occurs from the inner to the outer side of the wall. This is the basis of the multi-net growth hypothesis of Roelofsen and Houwink (1953). It is the inner portion of the wall which plays the greatest role in determining the permitted direction of expansion (Richmond et al., 1980). It is apparent that an alteration of the orientation of the deposition of cellulose microfibrils will lead to a change in the direction of cell expansion. This can be shown by various hormonal treatments which change cortical microtubule and nascent microfibril direction and subsequently alter cell polarity. For example, following treatment of pea epicotyls with ethylene, microtubules (e.g., Lang et al., 1982; Steen and Chadwick, 1981) and microfibrils (e.g., Ridge, 1973) switch from transverse to longitudinal and this results in a lateral swelling of the tissue (e.g., Eisinger and Burg, 1972). Examples have been cited above of shifts in cortical microtubules that occur in normal development. Many of these studies have an obvious bearing on the subsequent expansion direction of the walls involved, such as those involved in thickenings around stomatal pores (e.g., Busby and Gunning, 1984). However, in other cases, such as the deposition of alternate layers of longitudinally- and transversely- aligned cellulose in elongating collenchyma (Chafe and Wardrop, 1970), although microtubule control is heavily implicated, the relationship of such a wall structure to subsequent expansion is not so well delineated (discussed in Chapter 4).

Perhaps the most dramatic manifestation of the role of the direction of cellulose deposition in plant cytomorphogenesis occurs when a lateral
organ emerges. It must be circumferentially reinforced in order for it to elongate and so in certain regions it requires a cellulose orientation of 90° to that of the parent structure (Green, 1980). Green and co-workers have extensively investigated such changes during the formation of leaves and roots from the residual meristem of detached *Graptopetalum paraguayense* leaves (Green, 1980, 1984; Green and Brooks, 1978; Green and Lang, 1981; Green and Poethig, 1982; Hardham et al., 1980; Selker and Green, 1984). These studies have revealed that localised switches in the direction of cellulose deposition occur in the epidermis prior to the initiation of the new organ, so that reinforcement is, on the whole, normal to the overall direction of expansion. These changes are mirrored by cortical microtubule behaviour. Polarity changes also occur in the interior of the site of the new organ, but these appear to be less well defined and secondary to those of the epidermis (Selker and Green, 1984). Such discoveries illustrate that, in organogenesis, the controlling area is the biophysically important epidermis (Green and Poethig, 1982, see also Lyndon, 1982) rather than the interior tissue. However, as will become apparent in subsequent chapters, the polarity shift in wounded *D. stramonium* internodes apparently does not involve epidermal control.

From the above synopsis, it is apparent that microtubules play a major part in determining the shape of cells that constitute a plant organ. This role transcends areas from the determination of the division site through mitosis to the architecture of the cell wall. The cytoskeleton is of course important in other aspects of organ formation that have not been introduced here, such as differentiation events; for example, microtubular control of xylem bar formation (e.g., Hepler and Foskett, 1971).

In contrast to a significant proportion of the preceding discussion, it has been proposed that, in many types of wall, expansion can take place
in any direction and that microtubules play no part in the determination of
the structure of the wall. Briefly (this is further discussed in Chapter
4), the papers of Roland and colleagues (e.g., Roland and Vian, 1979;
Roland et al., 1982) and Neville and co-workers (Neville and Levy, 1984;
Neville et al., 1976) propose that many types of wall 'self-assemble' in a
series of gradually rotating layers - a helicoid. Such walls were
originally considered to be those with an obvious polylamellate
construction such as outer epidermal walls, but the proposition has been
extended to include cells such as parenchyma which are usually described as
fitting the multi-net growth hypothesis. Microtubules are assumed to have
no role in the self-assembly process. Walls of this type are termed
'dissipative structures' (Roland et al. 1982) and are thought to have the
ability to extend in both transverse or longitudinal directions (e.g., Reis
et al., 1982), the limit of expansion being reached when the original order
has been completely lost (Roland et al., 1982). Variation of the 'time
clock' (Roland et al., 1983) of deposition of the successive layers is
thought to be responsible for the different types of wall (such as
parenchyma or collenchyma) that occur and treatments such as incubation
with ethylene are thought to keep walls in a 'youthful' state (Reis et al.,
1982). Secondary walls, laid down after extension has ceased, are thought
to represent ordered helicoids not dispersed by extension.

Several objections have been raised to the hypothesis of helicoidal
self-assembly of walls (e.g. see Lloyd, 1984a; Preston, 1982; Wardrop et
al., 1979). A recent criticism is that the helicoidal self-assembly of
chitin microfibrils in insect cuticles (upon which the plant model is
based, see Bougiland, 1972) takes place in a proteinaceous matrix, whereas
the matrix substances of plant cell walls are mainly polysaccharides
(Robinson and Quader, 1982). The majority of the work supporting the
helicoidal hypothesis (which is a modification of the original alternating deposition of the ordered fibril hypothesis of Roland et al., 1975) is based on EM of transverse sections, mainly using the Thiéry (1967) stain for polysaccharides to visualise the cellulose microfibrils. However, the exact mechanism by means of which the silver proteinate stains is unclear and may well be physically based and so, 'The Thiéry staining procedure could under some circumstances be misleading', (Wardrop et al., 1979). Indeed, the original Thiéry (1967) paper, to which Roland and co-workers invariably refer for the method, hints at the unreliability of the stain by adding the caveat that 'Le protéinate d'Argent est un produit dont la composition n'est pas strictement définie. Nous avons utilisé le protéinate d'Argent fabriqué par les établissements Roques, Paris, qui nous a donné de bons résultats'. When interpretation of structure is at issue the definition of 'bons résultats' may well vary. Furthermore, interpretations of wall structure, when examining the thin slice of wall visualised in sections using EM, are subject to the physical problems of deducing microfibril layout (e.g., see Chafe and Doohan, 1972; O'Brien, 1972). The type of cells used as evidence in support of the helicoid model raise additional doubts. Wardrop et al. (1979) and Preston (1982) suggest that the cells considered to be parenchymatous by Roland et al. (1975, 1977) are in fact collenchyma. Also recent work by Emons (1982), Emons and Wolters-Arts (1983) and Sassen et al. (1981) focussing on the proposed helicoidal nature of cell walls and lack of microtubule control in root hairs can be criticised on the grounds that such cells are tip-growing. Wall deposition behind the tip can thus be regarded as secondary, hence extrapolation to the more typical plant cells undergoing surface growth may be tenuous. Indeed, many secondary walls are known to be helically arranged, i.e., possessing two or more layers in helices, but, in contrast
to the helicoidal organisation, each constituent layer is made up of similarly aligned lamellae of microfibrils and there are major switches in direction between each layer (e.g., see Preston, 1974; Roelofsen, 1959).

Lloyd (1984a) has attempted to reconcile the contrasting views of helicoidal self-assembly and microtubule control by proposing that interphase microtubule arrays are arranged in a helix of long, overlapping microtubules and that these form a variety of arrangements from compressed helices giving near-transverse order to more steeply-pitched forms resulting in nearly longitudinal deposition. Using immunofluorescent techniques Lloyd (1983, 1984a) has reported a helical arrangement of microtubules in root hairs. This does much to reinforce support for microtubule control in such atypical (tip-growing) cells, but such arrays have not yet been demonstrated in extending, primary walls.

In this thesis, the examination of the polarity change and the associated cell response to tissue wounding is reported. In the experimental system selected, a portion of the vascular cylinder is removed from the recently-matured internode of D. stramonium. The wounding induces previously quiescent, vacuolated, relatively simple, pith cells to undergo a predictable 90° shift in polarity (e.g., see Warren Wilson and Grange, 1984), dividing many times to generate a wound cambium, which ultimately restores a comparatively normal stem-like organisation (Warren Wilson and Warren Wilson, 1983).

The main aim of the work carried out was to document the initial stages of the wound response on a cellular and sub-cellular level in order to attempt to gain some further insights into the role of the cytoskeleton in plant development in general. The study concentrated on events leading up to the first round of the wound-induced divisions. It transpired that
this was the period of most dramatic protoplasmic reorganisation. This permitted investigation of factors involved both in the change of polarity and in early manifestations and determination of the division site. The relationship between PPBs and phragmosomes was studied.

Examination of the role of cell wall changes in the switch of polarity, using a range of light- and electron-microscopic techniques, was permitted by extension of the hand-carving techniques developed by Green for epidermal tissue undergoing a polarity change. This allowed the timing of wall deposition to be determined in relation to the first round of division. Unexpectedly, the deposition turned out to be polylamellate, offering an opportunity to analyse its structure in relation to the helicoidal model described above. It had not hitherto been possible to correlate the structure of walls and the cortical cytoskeleton in large vacuolated cells. The cells investigated were, in fact, so large that a novel combination of immunofluorescence, polarising, interference and EM became possible, permitting observations at several levels of analysis on individual cells.
CHAPTER TWO

WOUND RESPONSE IN GENERAL
2.1 INTRODUCTION

The control of the plane and timing of division and of the rate of cell enlargement is one of the prime determinants of form in multicellular plant tissues (e.g., see articles in Barlow and Carr, 1983; Lloyd, 1982). Most studies of division and morphogenesis in higher plants have concentrated on the small, densely packed cells of apical meristems where the simultaneous examination of a large number of active components is possible, especially at the ultrastructural level (e.g., Gunning et al., 1978a, b, c). As cells exit from their meristematic phase they generally become differentiated into specialised components which, in total, comprise the whole organ. Whereas in normal development they retain their peculiar characters, in response to wounding differentiated cells may be induced to re-differentiate into other cell types. Much work has been done in the investigation of these phenomena, especially the regeneration of vascular tissue in dicotyledonous stems (e.g., see Warren Wilson and Warren Wilson, 1983). There are two types of re-differentiation. By analogy with post-wounding phenomena in animals, these have been termed 'epimorphosis' and 'morphallaxis' by Warren Wilson and Warren Wilson (1983). Epimorphosis usually occurs in response to gross wounding, such as that inflicted in the present work, and involves the cells near the wound re-entering the mitotic cycle, dividing repeatedly and re-differentiating to reform the lost tissue (e.g., Warren Wilson and Grange, 1984). Morphallaxis, which usually occurs in response to small wounds, involves the cells surrounding the wound changing structure and function in order to replace the lost tissue without the addition of new material (e.g., Benayoun et al., 1975). In some cases cells achieve this directly, without an intervening mitosis (e.g., Hammersley and McCully, 1980), whilst in others one or more rounds of division may be necessary before re-differentiation occurs (e.g., Behnke
and Schultz, 1980). Hardham and McCully (1982a, b) have reviewed both types of response.

Most studies of anatomical changes associated with wound responses have employed light microscopy and have concentrated on events that are seen several days or more after wounding (e.g., Sussex et al., 1972). Only a few have examined details of the initial cellular responses (e.g., Kirschner and Sachs, 1978). Of the ultrastructural investigations, Hepler and Foskett (1971) examined xylogenesis, while Behnke and Schultz (1980) and Hardham and McCully (1982a, b) studied the regeneration of phloem around a wound. Yet documentations of early changes, especially where a change in cell polarity is later manifested, could provide significant information about the operational control of normal plant development.

Many of the differentiated cells involved in the resumption of meristematic activity in response to wounding are of especial interest because they are highly vacuolated. Division in vacuolated cells, whether in response to wounding or during normal development, has been little-studied in recent years. Once again, observation of the events involved could throw new light on morphogenetic processes, in that some of the features may be present in an exaggerated form as compared with small meristematic cells. To give one example, growth of a phragmoplast across a large vacuole for tens or even hundreds of µm away from the vicinity of the telophase nuclei, shows conclusively the independence of the phragmoplast from the spindle and could also reveal much more about spatial control than the same process in a small cell. Phragmoplasts in vacuolated cells develop within a cytoplasmic structure - the phragmosome - which is not detectable in densely cytoplasmic cells. The phragmosome, first named by Sinnott and Bloch (1940, 1941a, b)* is a raft of cytoplasm traversing the vacuole. It contains the nucleus and forms before mitosis in the plane of

*Porter and Caulfield (1958), whilst acknowledging that 'phragmosome' had already been coined by Sinnott and Bloch, used the term to describe densely-staining vesicles that they observed in cell-plate formation and Manton (1961) continued this misuse. As pointed out by Mahlberg et al. (1975), the Sinnott and Bloch usage should be preferred, even if only on a precedent basis.
the future new wall, remaining in place until the cell plate, which develops within it, fuses with the parental wall. The recognition of the phragmosome, long before electron microscopic studies of PPBs, was a clear demonstration that plant cells exert spatial control of cytokinesis prior to the onset of mitosis. Despite the realisation of the importance of phragmosomes, relatively few studies of them have been carried out (Bergmann, 1960; Brown and Dyer, 1972; Esau and Gill, 1965, 1969; Evert and Deshpande, 1970; Goosen-de Roo et al., 1980, 1984; Jones et al., 1960; Roberts and Northcote, 1970; Sinnott and Bloch, 1940, 1941a, b; Venverloo and Libbenga, 1981; Venverloo and Pronk, 1982; Venverloo et al., 1980, 1984; Yeoman et al., 1970). Similarly, there have been few descriptions of PPBs, which have been much studied in more dense tissues (e.g., Gunning, 1982a), in highly vacuolated cells, either prior to normal (Esau and Gill, 1969; Evert and Deshpande, 1970) or wound-induced mitoses (Hardham and McCully, 1982a, b). Only two studies to date have been directly applied to the relationship between PPBs and phragmosomes in vacuolated cells and then in epidermal explants in vitro (Venverloo and Pronk, 1982; Venverloo et al., 1980).

In order to investigate the details of the wound response of *D. stramonium* pith cells a variety of techniques was utilised. EM was required for the visualisation of PPBs, but thin sections, especially of such large cells, were not ideally suited to the identification of phragmosomes. Thus, the bulk of the anatomical changes that occurred, were elucidated at the level of the light microscope using hand sections and sections of glycol methacrylate- (GMA) embedded material. The former involved conventional hand-sectioning techniques, except that the sections were cut rather thickly owing to the large size of the cells. Similar sections were also examined using scanning electron microscopy (SEM).
standard GMA procedure for light microscopy was initially used, but problems of cracking and folding were encountered when sectioning the large vacuolated cells, especially when whole transverse sections of the wound area were used. A recent modification to methacrylate resin, employing Sorvall Embedding Medium (SM) was utilised to alleviate these problems. This medium incorporates benzoyl peroxide as a free radical initiator (rather than as a catalyst) to polymerise 2-hydroxyethyl methacrylate, a process which is accelerated by the addition of N,N-dimethylaniline, thus obviating the requirement for ultraviolet (UV) radiation (see Robinson, 1982). Once a few initial problems had been overcome, SM proved superior to GMA.

The large-scale morphological changes that occur during the wound response of *D. stramonium* pith cells, especially the factors involved in the re-differentiation of the reformed tissue, have already been studied in considerable detail (see Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1983 and references therein). Using the combination of techniques outlined above, this chapter reports the extension of these data to the cellular level with especial regard to the establishment of the division site. Information is presented for the first time on the relationship between PPBs and phragmosomes in differentiated, vacuolated cells undergoing a change in polarity and dividing in response to wounding in vivo.
2.2 MATERIALS AND METHODS

2.2.1 Germination and growth of plants

Seeds of *Datura stramonium* L., either from an original natural population (collected locally in 1980 and kindly supplied by Dr. A. Gibbs, R.S.B.S., A.N.U.) or from the first generation of plants from this wild source, were germinated in a growth cabinet with a short day (8 or 10h) and a high diurnal range (usually 30/20°C) environment. This régime overcame the initial low germination rate, a problem which is common with *Datura* species, see Monaghan and Felton (1970) also pers. comm. Drs. P. Dart, A. Gibbs and P. Warren Wilson, Mr. R. Lamb (R.S.B.S., A.N.U.) and Ms S. Dircks (Botany Dept., A.N.U.). Even a batch with a germination rate of less than 1% (kindly supplied by Ms S. Dircks) gave reasonable levels of germination when the above protocol was used together with an initial soak in 0.2% (w/v) KN03 - as recommended by Mr. R. Pullen, C.S.I.R.O., Canberra. After germination, seedlings were spiked out individually into 10cm pots and transferred to a naturally-lit glasshouse with a summer temperature range of 18-20/28-29°C and a winter of 12-15/23-26°C. Plants not used for seed collection were kept in a vegetative state by excising flower-buds.

2.2.2 Wounding

Wounding was inflicted, using a technique similar to that employed by Warren Wilson and Warren Wilson (1961a), upon plants where the first crotch had fully formed. Usually this took place 4 to 5 weeks after the spiking-out of the seedlings. Prior to wounding a wooden swab-applicator was tied against the stem with flexible grafting tape to prevent stem curling. Starting at the petiole of the leaf immediately below the crotch, a vertical slab of the stem approximately one-third of its width and 2cm in length was excised (Fig. 2.1a, b) using a sterile scalpel-blade. In most
experiments, and unless otherwise stated, tissue was harvested before wounding, immediately after wounding (day 0), and at daily intervals thereafter for 7 days (days 1-7).

2.2.3 Light microscopy of the wound response

For surface views of the wound a Wild M400 Photomakroskop was used. Otherwise, all light microscopy was carried out using Köhler illumination or differential interference contrast (DIC) on a Zeiss Photomicroscope III. For stained plastic sections (Section 2.2.3.2) DIC optics were sometimes modified to use amplitude rather than phase differences (A-DIC) to generate contrast (David and Williamson, 1971). This was accomplished by setting the prism at its darkest position and rotating the polariser. Micrographs were made on Pan-X or Plus-X Kodak film.

2.2.3.1 Hand sections

Longitudinal and transverse hand-sections were cut, under fixative, from unwounded tissue as well as from stems 8h and days 1, 2, 3, 4 and 7 after wounding. The fixative was either standard EM fixative (Section 2.2.5) or 5% (v/v) glutaraldehyde (Polysciences Inc., Warrington, Pa, 18976, U.S.A.) only, in buffer (25mM potassium phosphate at pH 6.9 was used in all the experiments described in this chapter). After fixing at room temperature (RT, approximately 22°C), for a minimum of 2h and a maximum of overnight (12 to 18h), sections were gently rinsed and mounted in buffer under 'Valap'-sealed (Mole-Bajer and Bajer, 1968) coverslips for examination, either following staining in 0.05% (w/v) toluidine blue (Gurr, BDH Chemicals Ltd., Poole, England) in benzoate buffer at pH 4.4 (O'Brien and McCully, 1981) or directly. In addition, in order to facilitate examination of 'live' cells, some day 7 sections were cut and mounted,
without fixation, in buffer or the fluorocarbon Medifluor Fc-47 (Minnesota Mining and Manufacturing Co., St. Paul, Minnesota, 55101, U.S.A.).

2.2.3.2 Embedded material

Complete transverse sections approximately 2mm thick were cut under standard EM fixative (Section 2.2.5). Some were dissected further into cubes, each having an orientation mark on the opposite face to the wound in order that tissue axes could be later recognised. After gentle vacuum infiltration (three times over an hour), the tissue was fixed overnight, rinsed in buffer and subsequently processed for embedding in GMA or SM (Du Pont Co., Newtown, Connecticut, 06470, U.S.A.).

For the former, samples were gradually brought into 2-methoxyethanol and, with one change in each solution, through ethanol and n-propanol into n-butanol over a minimum of 4h. The n-butanol was gradually replaced by the monomer-mix and the samples infiltrated for 2 to 4 days with daily changes of solution. The monomer-mix comprised 95ml GMA, 5ml polyethylene glycol 200 (PEG 200; Koch-Light Laboratories, Coinbrook, Bucks., England) and 1.1g benzoyl peroxide (BDH Chemicals Ltd., Poole, England). Polymerisation was accomplished using UV radiation under flushing nitrogen at 4°C for 24h.

For embedding in SM, tissue was either processed into n-butanol, as for GMA, or dehydrated through an ethanol series prior to infiltration with the 'A + B' mix over a period of 2 to 3 days with twice-daily changes. Polymerisation in the 'A+B+C' mix was carried out for a minimum of 8h at 4°C under nitrogen. (It was found that polymerisation at RT, as recommended by the Sorvall instruction manual, occurs too rapidly, with excessive heat production. At 4°C the reaction slows down to an acceptable rate, but oxygen—an inhibitor—has to be excluded to ensure good, even
blocks.) To counter desiccation, when stored for long periods, SM blocks were sometimes placed above a saturated solution of \((\text{NH}_4)_2\text{SO}_4\) in a desiccator. This gave a relative humidity of 81%, which had been found to give the best block consistency in trial experiments using \(\text{H}_2\text{SO}_4\) solutions to vary moisture content. Longitudinal and transverse sections approximately 2 \(\mu\text{m}\) thick were cut on a Sorvall JB4 microtome (Du Pont Instruments) using 12mm glass knives, dried onto glass slides and stained using 0.1% toluidine blue in benzoate buffer at pH 4.4. Some sections were stained for calcium oxalate by flooding with a fresh 1:1 mixture of 30% (v/v) \(\text{H}_2\text{O}_2\) and 5% (w/v) \(\text{AgNO}_3\) and illuminating them with a 60 watt lamp at a distance of 20cm for 15min (a modification of the Pizzolato method used by Silver and Price, 1969).

2.2.4 Scanning electron microscopy

For SEM of internal tissue components, transverse sections 2 to 3mm thick were cut under fixative comprising 2.5% (v/v) glutaraldehyde in buffer, vacuum-infiltrated and fixed overnight prior to rinsing and 2h post-fixation in 1% (w/v) \(\text{OsO}_4\) (BDH Chemicals Ltd., Poole, England) in buffer. After a further rinse, samples were gradually brought into acetone, where they were cut in half transversely under the solvent, in order to expose a new, fixed surface for examination. A taper was also cut in order to facilitate specimen orientation. Specimens were critical-point dried from fresh acetone and \(\text{CO}_2\) in a Bomar SPC-1500 critical-point drier and mounted on copper studs using conducting paint (Ladd Research Industries Inc., Burlington, Vermont, U.S.A.). Mounted samples were sputter-coated for 4min with gold using a Polaron E 5000 SEM Coating Unit operating at 40mA and 1.2kV (giving a coat approximately 40nm thick) prior to examination and photography using an Hitachi HHS-2R scanning electron microscope operating at 15kV.
2.2.5 Transmission electron microscopy

In most experiments, tissue was cut into cubes, slightly smaller than as used for light microscopy (Section 2.2.3.2), under standard EM fixative. This comprised 2.5% (v/v) glutaraldehyde and 2.0% (w/v) paraformaldehyde (Ega-Chemie 7924 Steinheim/Albuch, West Germany) in buffer. After vacuum infiltration, overnight fixation, rinsing, post-fixation and dehydration through an acetone series as for SEM (Section 2.2.4), samples were gradually infiltrated with Spurr's resin (Spurr, 1969) prior to polymerisation at 70°C. 2 µm survey sections were stained with 0.5% (w/v) toluidine blue in sodium benzoate at pH 11 (O'Brien and McCully, 1981). Silver or gold sections for EM were cut on a Reichert OMU3 ultramicrotome, using a Du Pont diamond knife, collected on Formvar-coated 2mm slot grids, stained with 10% (w/v) uranyl acetate in 50% ethanol and lead citrate (Reynolds, 1963), prior to examination using Hitachi H-500 or H-600 electron microscopes operating at 75kV.

Other fixation régimes employed included the addition of 1.0, 0.5, 0.2, 0.1 or 0.01% (w/v) caffeine (Sigma, PO Box 14508, St. Louis, Mo, 63178, U.S.A.) to 2.5 or 0.5% (v/v) glutaraldehyde (12h fixation) and to rinsing solutions (Mueller and Greenwood, 1978). In some cases 0.3mM phenylmethylsulphonyl fluoride (PMSF), 1 mg/ml leupeptin and 5mM ethyleneglycol-bis (β-amino-ethyl-ether)-N,N'-tetra-acetic acid (EGTA) (all Sigma) were added to the standard fixative and, in others, glutaraldehyde or paraformaldehyde only were used in an otherwise standard protocol.
2.3 RESULTS

2.3.1 Embedding media for light microscopy

GMA was used in early experiments, but, once initial polymerisation problems were overcome, SM was preferred. Disadvantages of the GMA were that it tended to crack and fold, especially with large block faces, it stained to a certain extent with toluidine blue and it autofluoresced at the emission wavelengths of fluorescein and rhodamine. By contrast SM sectioned easily, hardly stained with toluidine blue and did not autofluoresce. Although SM blocks were more prone to desiccation during storage (presumably owing to the water-retention capacity of the PEG 200 in the GMA), this could be easily overcome by placing the blocks in an atmosphere of 81% relative humidity prior to sectioning. SM blocks stored for long periods in Newcastle, N.S.W., where the climate is much damper than in Canberra (Plumb, 1977), tended to absorb water and had to be dried prior to sectioning (Dr. C.E. Offler, University of Newcastle, pers. comm.). Both SM and, to some extent, GMA adhered only weakly to waxy (hydrophobic) surfaces. This was observed in cuticles of *D. stramonium* stems (e.g., Fig. 2.17) and apices of *Graptopetalum paraguayense* (not illustrated) as well as in anthers and seeds of some *Eucalyptus* species (Ms A. Forbes, R.S.B.S., A.N.U., pers. comm.) and could be partially alleviated by gently brushing the surface of the specimen with a detergent solution just before fixation. Although SM was initially used with the n-butanol dehydration procedure, similar results were obtained in later experiments with the simpler ethanol series.

2.3.2 Fixation for electron microscopy

The standard régime gave reasonably good preservation in most cells, especially considering their highly-vacuolated nature. In general, there
was only slight plasmolysis and a little extraction of the cytoplasmic ground substance. However, as with all protocols used, the quality of fixation sometimes varied from cell to cell (e.g., Fig. 4.31) and, occasionally, to some extent, even within cells. The addition of caffeine has been used to enhance cytoplasmic preservation in vacuolated cells by precipitating phenolic compounds (Mueller and Greenwood, 1978). Although \textit{D. stramonium} contains alkaloids (e.g., see Leete, 1959; Waller and Nowacki, 1978), no osmiophilic globules were observed when caffeine was added and thus, despite the improvement of chloroplast preservation in some cells, it was not used routinely owing to greater extraction of the cytoplasmic ground substance than with the standard protocol. This extraction may have been a consequence of using glutaraldehyde alone, as neither it nor paraformaldehyde gave as good a preservation when used separately as when the two were used together. The addition of protease inhibitors was found to enhance fixation of highly-vacuolated cells in bracts of \textit{Ornithogalum umbellatum} (Ms C. Busby, R.S.B.S., A.N.U., \textit{pers. comm.}). However, this was not found to be the case for \textit{D. stramonium}.

2.3.3 Pith cell anatomy before wounding

Transverse plastic- (Fig. 2.3) and hand- (Fig. 2.4) sections as well as SEM vistas (Fig. 2.5) reveal the large, highly-vacuolated, relatively thin-walled, 5, 6 or 7-sided polygonal shape of the stem pith cells in the normal, unwounded (and day 0) state. Triangular intercellular spaces and an increase in wall thickness are also apparent at most cell junctions, where SEM views (Fig. 2.5) often show the side/side and end/side wall borders, within the same cell, to be fairly abrupt. The longitudinal continuity of intercellular spaces is observable in hand sections (Fig. 2.7) (cf. Sorokin, 1966). These, together with plastic sections (Fig.
2.6), also illustrate the elongate nature of the cells, with side and end walls respectively parallel and normal to the long axis of the plant. However, some end walls are obliquely oriented.

Each cell possesses a single, flattened, ellipsoidal nucleus (Figs. 2.5 and 2.9) which is not associated with the major cell organelles to any great extent and, although most easily observed against end walls in transverse sections (Fig. 2.4) and side walls in longitudinal sections (Fig. 2.6), lies against any wall in the cell. This is in contrast to the amyloplasts which preferentially lie near the basal end wall (Fig. 2.7). Apart from such objects the cytoplasm is thin (Figs. 2.9 and 3.3) and barely visible in the light microscope. It is strictly cortical. In no cases were trans-vacuolar strands observed using any of the techniques executed. Assuming an average pith cell to be 250 µm long and 100 µm in diameter and the cytoplasm to be an average of 150 nm thick, it only occupies less than 1% of total cell volume. In the vast majority of cells the remaining volume was taken up with, seemingly structureless, vacuole. However, some cells were packed with crystals (Figs. 2.8a, b). Their general shape, appearance in the electron microscope (Fig. 2.10), birefringence (not illustrated) and the precipitation of silver by the Pizzolato method (Fig. 2.8a) strongly indicate that they are composed of calcium oxalate.

EM reveals that the side walls are transversely reinforced with cellulose (Fig. 3.3), whilst the end walls (Fig. 3.16) have only local groups of similarly-aligned microfibrils with no overall order (pith cell wall architecture is detailed in Chapter 3). Congruent microtubules were seen in both cases (Figs. 3.3 and 3.16). Microtubules also displayed links between themselves (Fig. 2.11), probably microtubule-associated proteins (MAPs), and to the plasma membrane (Fig. 2.12). The thin cytoplasm also
contains the structures, other than those previously described, typical (Gunning and Steer, 1975) of mature higher plant cells viz.: endoplasmic reticulum (ER), with associated polyribosomes, underlying a considerable portion of the wall (Fig. 3.16); dictyosomes, with their vesicles appearing to fuse with the plasma membrane (Figs. 3.3); mitochondria (Fig. 2.7); lipid bodies (Fig. 2.9); microbodies (not illustrated) and putative actin filament bundles (Fig. 3.16).

2.3.4 Gross anatomy of the wound response

The gross anatomy of the wound response is shown in surface views of a single specimen (Figs. 2.2a-r) and complete transverse sections of days 1 to 6 wounds (Figs. 2.13 - 2.18). The wound was made at a time when the internode had just finished longitudinal expansion and where subsequent increase in girth would have occurred in normal development only by expansion of the vascular cylinder and the cells exterior to it. The pith, apart from developing a hollow centre, would have remained unchanged. During the wound response, little or no increase in length occurs (cf. Fig. 2.2b with 2.2r). Minimal radial expansion is apparent in surface views (Figs. 2.2c, d) and in transverse sections (Figs. 2.13 - 2.15) until cell divisions start - usually around days 3 or 4 (Fig. 2.16). Once initiated, many rounds of cytokinesis take place in the pith below the wound (Figs. 2.17 and 2.18). There is, however, a non-dividing, moribund layer immediately under the cut surface that is usually 1 or 2 cells deep (e.g., Fig. 2.17), but occasionally encompasses several layers (e.g., Fig. 2.18). Associated with the resumption of meristematic activity, is a large increase in radial expansion apparent both in sections (Fig. 2.18) and surface views (Figs. 2.2e-h). The main focus of interest in this study is these responding pith cells, where the vast majority of the initial and
most of the later divisions are parallel with the wound. However, periclinal divisions also occur in the outer cortex, but they soon cease and no cambium forms. Divisions in the pith appear to start adjacent to the remaining vascular tissue (Fig. 2.16). In some cases many divisions occur in this area (Fig. 2.17), possibly where internal phloem, a feature of solanaceous species (Metcalfe and Chalk, 1950), remains near the cut surface. On the other hand, little or no mitotic activity occurs in the vicinity of the wound in the epidermis, collenchyma or mature vascular tissue (Figs. 2.17 and 2.18). The vascular tissue, as seen in surface views, is pushed outward (e.g., Fig. 2.2g) by the divisions within the pith during the initial period, when the increase in radial expansion results in the wound becoming markedly wider than the tissue above and below it. However, upon cambium formation (expected to be around days 9 to 11 in this tissue, Evans, 1979; Warren Wilson and Grange, 1984) the surface of the wound gradually smooths out and the difference in the width of the wound compared with the adjacent stem declines (Figs. 2.2f-r). Hand-sectioning of the wound used for these surface studies revealed a typical response (see e.g., Warren Wilson and Warren Wilson, 1983) with the formation of a comparatively normal stem-like organisation (not illustrated). This was also the case with two other wounds studied in the same manner.

2.3.5 Pith cell anatomy after wounding

The first stage of the cytoplasmic response is apparent a few hours after wounding. It can be observed in cells ranging from those located immediately beneath the cut surface to those several files back from it. Amyloplasts, which may be associated with fibres (Fig. 2.27b), cluster around the nucleus (Fig. 2.19). By day 1 these 'organelle enclustered nuclei' (Figs. 2.50 and 2.51) are larger and less flattened (Figs. 2.34 and 2.50) than before wounding and possess - usually one per nucleus - swollen
nucleoli (Fig. 2.50). They have also moved to a position half-way up the side of the longitudinal wall (Fig. 2.34) nearest to the wound (Figs. 2.29 and 2.32) - the 'wound wall'. This movement, termed 'traumatotaxis' by Tangl (1884), must be accomplished by the nucleus moving around the walls of the cell, as no (cf. below) trans-vacuolar strands were seen and all nuclei lay against walls. In addition, the position of the organelle-enclustered nuclei at all points of the cell periphery (Fig. 2.19) provides further evidence that amyloplast movement occurs before, or very early during, nuclear migration. The density of nuclear pores, as judged by their frequency in cross-sections, increases over the pre-wounding condition (cf. Fig 2.9 with Figs. 2.49 and 2.50). Microtubules are seen associated with traumatotactic nuclei (Figs. 2.49 and 2.51). However, owing to the difficulty of determining which nuclei were actually migrating in such images, it is not clear whether microtubules are involved in the nuclear movement. Microtubules may be represented in the network of enveloping filaments seen ensnaring some traumatotactic nuclei in images obtained by SEM (Fig. 2.27c), although such a mesh could equally contain filamentous (F-) actin. However, actin filaments were not observed around nuclei using EM, but, notwithstanding its seeming preservation in fixed material (Fig. 3.16), its visualisation is difficult. Also apparent at this stage is the collapse of cells immediately under the cut surface, although traumatotaxis may have occurred in some of them (Fig. 2.29).

The next facet of the response, most easily visualised in hand sections (Fig. 2.20), is the formation of thin trans-vacuolar strands emanating from the nucleus. The nucleus then moves through the vacuolar void (Figs. 2.27d and 2.33), supported by the cytoplasmic strands, to reside in the spatial centre of the cell (Figs. 2.20 - 2.22 and 2.35), thus forming a 'systrophe' or cytoplasmic star. The term systrophe is used in
this report to refer to a nucleus, with enclustering organelles, suspended by fine radiating trans-vascuolar cytoplasmic strands approximately in the spatial centre of the cell. This utilisation of the term systrophe is similar to its usage to describe organelle aggregation around a nucleus in response to plasmolysis (Germ, 1932a, b, c; Weidinger, 1980a, b; 1982) or illumination (Senn, 1908; Weidinger and Ruppel, 1985)*. In thick hand-sections, streaming was observed in the radiating anastomosis (not illustrated), often continuing for one or two hours after cutting, a period not increased by the use of Medifluor Fc-47. Actin was not observed in such strands in the EM, but their orientation and relatively small size when compared with the cell meant that extensive lengths of strand were rarely seen and so actin filaments may well have been present, although not visualised. On the other hand, microtubules, which are more readily observable, were present (Fig. 2.64).

Strands in adjacent cells were often seen to lie opposite each other (Fig. 2.23). This is also the case at the next stage of the wound response; the formation of phragmosomes. These are best recognised in hand sections (Fig. 2.25), as considerable sectioning is required in order to identify them conclusively in embedded material. Even in hand sections, changing the plane of focus in order to observe the whole depth of the cell is required to separate them from recent divisions. Phragmosomes

*Weidinger and Ruppel (1985) imply that this term was first used by Senn (1908). However, Senn cites Schimper (1885) as the originator of the expression, as does Jackson (1928) in his 'Glossary of botanic terms'. Indeed, in an earlier paper, Weidinger (1982) lists the Schimper paper, but without an original reference. In fact, Schimper (loc. cit.) used the word systrophe both alone and also in conjunction with the prefixes apo-, epi- and para- to describe various forms of clustering by organelles within cells. Senn (loc. cit.) only used the term systrophe and restricted its usage to clustering of organelles around nuclei. 'Systrophe' is not in the Oxford English Dictionary (although, curiously, the botanical uses and etymology of apo- and epi-systrophe are listed), but Jackson (loc. cit.) describes it as a substantive and it is used as a noun in this thesis. Similarly, also following Jackson, 'systrophic' is used as an adjective.
presumably are formed by coalescence of the radiating arms of the systrophe; indeed other strands are often left in addition to the main structure.

The first round of division, which generally occurs on days 3 or 4, is striking in two respects. Firstly, the vast majority of the divisions are parallel with the wound surface (Figs. 2.23, 2.25 and 2.31). Secondly, divisions in adjacent cells often meet at 4-way junctions (Figs. 2.23 and 2.25), i.e., new walls in adjacent cells abut each other exactly, a situation usually avoided by plants in normal development (Sinnott and Bloch, 1941b). This lining-up of divisions may traverse 3 or 4 cells, but generally not much further. Whilst there are earlier less extensive manifestations, (Fig. 2.33), it becomes most apparent at this stage that a considerable thickening of the cell walls has occurred. That this deposition is a wound response and not merely a reflection of variation between specimens, is well-illustrated by taking the collapsed cells from immediately under the cut surface, which have the thin walls of their original pre-wounding state and thus act as an internal control, and comparing them with those two or three files back which have undergone a cytoplasmic change (Figs. 2.30 and 2.31). There is also a spatial gradient in the wound response. It is common to see most of the above stages in one vista (e.g., Figs. 2.27a and 2.31). From these views it is apparent that, in addition to it happening in the layer immediately under the wound, delayed cytoplasmic death usually occurs in the succeeding one or two layers, although the cells remain fairly rigid and often illustrate the initial stages of the response. Their delayed death seems to follow suberisation, as apparent in electron microscopic images of such cells (Fig. 4.31), and their autofluorescence characteristics (see Section 5.3.1). Interestingly, it is the cells immediately under the moribund
layer that eventually show the greatest number of divisions (Figs. 2.44 and 2.45).

Timing of the wound response varied from plant to plant and experiment to experiment. However, the sequence of events was always identical and temporal differences appeared to mainly occur in the prelude to the first division, subsequent cell cycle durations being much shorter than the first. A day after the appearance of the first new walls the second round of cytokinesis has occurred in many cells. A similar pattern of events is repeated in these and subsequent divisions, although not preceded by traumatotaxis. Cytoplasmic strands appear traversing the newly-formed cell from the nucleus lying against the recent cross-wall (Figs. 2.36 and 2.37). The nucleus is often associated with amyloplasts, but these have generally become reduced in size, presumably owing to mobilisation of their starch reserves. After star formation (Fig. 2.38), a phragmosome develops (Fig. 2.37) in which division proceeds (Figs. 2.39 - 2.43). Repeated divisions occur over the next few days. Many of them are parallel with the wound (Figs. 2.24 and 2.28), but an increasing proportion are anticlinal or oblique (Figs. 2.26, 2.28, 2.47 and 2.48).

Cells containing calcium oxalate that are within the meristematic zone also divide and crystals are found in most, if not all, of their progeny (Fig. 2.46). However, only a few crystals are apparent in each cell, thus little or no replication of them can have occurred during the division of the mother cell.

The new walls are in direct continuity at 4-way junctions during some of these later divisions (Figs. 2.28, 2.44, 2.45 and 2.52). These junctions sometimes develop into new intercellular spaces (Figs. 2.37, 2.44 and 2.45). Divisions are generally confined to the first two or three files of responding cells, although cells beyond this cytokinetic zone
often show traumatotaxis and wall-thickening. Thus, the initial stimulus, as manifested by nuclear migration, occurs throughout a considerable depth of cells, but the complete response only occurs in some of them. Concomitant with the rapid divisions, a radial expansion of the tissue occurs. This is made most apparent by changes in the outlines of the mother-cell walls, many of which are still readily discernible at this stage, having elongated considerably in a direction normal to the wound (Figs. 2.24, 2.28, and 2.44 - 2.48).

2.3.6 Pre-prophase bands, phragmosomes and mitoses in the wound response

Phragmosomes are consistently present in the prelude to the first and subsequent rounds of wound-induced division. Thus, investigation of the occurrence of the other main identified predictor of the plane of plant cell division, the PPB of cortical microtubules, was obviously required. Cortical microtubules were examined with this in mind and also to study their role in the control of the wall thickening that occurs prior to the onset of divisions (see Chapter 4).

The large vacuolated nature of the cells involved made their examination difficult. Only a few cells occur in any given electron microscopic section and transverse serial-sectioning of an average cell would take over 3,000 thin sections. Thus, the probability of finding a feature of interest, especially if it is ephemeral, is small when a comparison is made with those meristematic zones which are usually studied. For example, an *Azolla* root primordium, containing around 500 cells, would fit inside one of the larger *D. stramonium* pith cells. The large cell size also made correlation of fine structure with large scale organisation difficult, for example, the determination of whether a particular observed microtubule pattern was in a cell whose nucleus was
near an end wall or was in the spatial centre of the cell. These hurdles could be partially overcome by the technique of examining 2µm sections singly in the light microscope and, on reaching a feature of interest, trimming the block and sectioning for EM. However, localisation of phragmosomes in such sections was still difficult and the identification of pre-prophase nuclei was virtually impossible.

Two cells at the correct stage for elucidating the relationship of phragmosomes to PPBs were observed. The day 5 pith cell shown in Figure 2.53, although relatively small (around 25µm in diameter), has thickened walls typical of the wound response (Chapter 4) and lies in the midst of the mitotic zone; nearby cells having recently divided parallel to the wound (not illustrated). Cortical microtubules, longitudinally aligned, only occur in two limited regions (Figs. 2.55 and 2.57) which are opposite each other, parallel to the wound, at the base of the large raft of cytoplasm which traverses the cell. One band has an average of 16 microtubules over a distance of approximately 7µm, whilst the other has 25 microtubules over 11µm (both averaging 2.3 microtubules µm⁻¹). The microtubule profiles, 1-3 layers deep, presumably represent cross-sections of an encircling, longitudinally aligned, periclinal PPB.

The cytoplasmic bridge is a phragmosome, but as is commonly observed (Section 2.3.5), other supporting strands are also present. The cytoplasm constituting phragmosomes and other cytoplasmic strands, as visualised throughout this study, appeared to be no different from that of the cortex, containing the same organelles. However in an analysis of over 20 serial sections, no trace of microtubules was seen in the supporting strands (Fig. 2.54) whilst many were observed between the PPB and the nucleus (Fig. 2.55). Microtubules were also found around the nucleus and, especially, associated with the nuclear envelope (Fig. 2.56). Thus, this cell may well
have been at the transition stage between PPB and spindle formation (cf. Wick and Duniec, 1984). Although microtubules were seen near the nucleus at the traumatotactic stage (Section 2.3.5), considerably fewer were present and they were not associated with the nuclear envelope to such an extent (Figs. 2.49 and 2.51).

The day 4 cell shown in longitudinal section in Figure 2.59 was undergoing the second round of mitosis having divided once, as had its neighbours, parallel with the wound. Some radial expansion had also occurred. One of the daughter cells had just started cell plate formation parallel to the cut surface and the other cell was at the systrophic stage. The few cytoplasmic anchoring points in the dividing cell were presumably in some form of phragmosome. On the other hand, the systrophe-containing cell had an obvious star-shaped central mass of cytoplasm around the nucleus, plus surrounding radiating cytoplasmic strands (Fig. 2.58) and therefore had not formed a phragmosome. This is an important observation as the cell with the 'star' formation possesses a PPB, predicting division parallel with the wound. No cortical microtubules were observed, except at the base of two of the strands (Figs. 2.61 and 2.62) which are adjacent to the end walls of the cell and opposite to each other. One band contained 40 microtubules over approximately 12 μm, the other around 34 microtubules over 10 μm (both averaging around 3.3 microtubules μm⁻¹). The microtubules, 1-2 layers deep, have been sectioned transversely and so the PPB is aligned in the same manner as that seen in the cell in Figure 2.53.

Occasional microtubules were found in several of the cytoplasmic strands (Fig. 2.64) and not just those adjacent to the PPB, but these were much fewer in number than were observed between the PPB and the nucleus in the pre-prophase cell already described. None of the non-PPB strands contained microtubules at their bases (Fig. 2.60). Microtubules were found
around the nucleus (Fig. 2.63), but not as closely associated with the nuclear envelope as in the previous example (Fig. 2.56).

Subsequent mitosis, as evidenced by the relatively few examples seen using EM, followed published general descriptions (e.g., Gunning and Steer, 1975), with one striking difference. Up to cell plate formation, the mitotic figure occupied a very small volume of cytoplasm compared with the total size of the cell (Figs. 2.59 and 2.70).

Organelles are generally excluded from the spindle zone (Fig. 2.65) and are fairly equally distributed on both sides of it, presumably to ensure that each daughter cell receives a share of the cytoplasmic components. In addition to the large numbers of microtubules; many vesicles, ribosomes and some rough ER were also present in the spindle (Fig. 2.65). Microtubules focus upon kinetochores with sister chromatids attached to microtubules from opposite poles (Fig. 2.65). Phragmoplast formation was seen to start whilst spindle microtubules were still present and prior to the complete reformation of the daughter nuclear envelopes (Figs. 2.66a, b). Once initiated, the phragmoplast has to traverse a long distance across the vacuole in order to fuse with the parental walls at a site presumably predicted by the PPB and it accomplishes this by extending centrifugally through the phragmosome (Figs. 2.42, 2.43, 2.67, 2.68a and 2.70). Microtubules, with associated vesicles, are only found at the growing margins of the cell plate. They disappear upon vesicle consolidation into the forming cell plate. Owing to an uneven positioning of the mitotic figure, the phragmoplast often meets and fuses with one parental side wall well in advance of the other (Fig. 2.70), as exampled in Figure 2.68a where there is a gradient in cell plate maturation from one side to the other. Also, in these elongate cells a centrally-positioned periclinal cell-plate will encounter the side walls well in advance of the end walls. ER runs parallel with the newly-forming wall and penetrates through it at several points (Fig. 2.68b). As the new wall thickens these sites become apparent as plasmodesmata (Fig. 2.69).
2.4 DISCUSSION

The various techniques employed facilitated the first detailed, although still incomplete, synthesis of the anatomical and ultrastructural changes in highly vacuolated cells undergoing a switch in polarity and a resumption of meristematic activity in vivo. For example, hand-sectioning provided information on live cells and the three-dimensional organisation of the cellular response, especially that of the fine cytoplasmic strands that would have been difficult to examine in sectioned material. This information was supplemented by SEM and the development of SM for routine use allowed a more detailed, but also extensive, examination at the level of the light microscope. Areas in need of further study by electron microscopic methods were thereby pinpointed, thus alleviating the problems of applying electron microscopic methods to the large, highly vacuolated cells.

The quiescent, but non-moribund, nature of the pith cells prior to wounding, with their flattened, amitotic nucleus lying in the thin cortical cytoplasm, is similar to that observed in other vacuolated cells used for related studies (e.g., Sinnott and Bloch, 1941a; Venverloo et al., 1980). Although the epidermal cells of Nautilocalyx used by Venverloo et al. (1980) always possessed one or more thin cytoplasmic strands traversing the vacuole, no such strands were observed in the current system. The possible role of putative nuclear anchoring strands (see Lloyd and Barlow, 1982) in the wound response is discussed below and it may be that they occur in the pith cells prior to wounding, but are not visualised or preserved (cf. Blackmore et al., 1984; Lloyd et al., 1982). In this regard it is noteworthy that, while nuclei are found against all walls, suggesting some form of anchoring (Lloyd and Barlow, 1982), amyloplasts tend to be located at the base of the cells.
Among the initial responses to wounding that have been observed in plant tissues, are an increased rate of cytoplasmic streaming (Miehe, 1901) and a rise in temperature (Ziegler, 1955) in the vicinity of the wound. It would be impractical, if not impracticable, to make observations on these phenomena during the reaction to wounding of *D. stramonium* pith cells. Thus, the first manifestation apparent in the present system was the clustering of the amyloplasts around the nucleus. Plastid clustering and nuclear enlargement were also observed in parenchyma cells of *Pinus radiata* hypocotyls to be the first stage of the change in polarity leading to the development of a meristematic locus and adventitious root formation (Smith and Thorpe, 1975). In a time-lapse study of division in vacuolated suspension culture cells of *Acer*, Roberts and Northcote (1970) also noted that plastids accumulated at both poles of nuclei positioned against walls prior to the onset of mitosis. However, in similar investigations of vacuolated epidermal cells of *Nautilocalyx* explants (Venverloo and Libbenga, 1981; Venverloo et al., 1980 and 1984) plastids accumulated around the nucleus only after star formation and they dispersed again prior to the onset of mitosis. These observations contrast with the events in *D. stramonium*, where the amyloplasts remained encircling the nucleus throughout its migration and only became associated with the two nuclear poles during mitosis. The mechanism of this organelle movement appears not to have been studied in detail, although close association between organelles and microtubules was noted by Roberts and Northcote (1970). Scanning electron microscopic images in the current study show putative cytoskeletal elements around organelle-enclustered nuclei. The reasons for enclustering are also obscure. Apart from ensuring an equitable division of organelles among the daughter cells upon division, the proximity of organelles such as amyloplasts and mitochondria to the supply of mRNA from
the nucleus may facilitate the increase in protein synthesis and respiration observed in cells responding to wounding (e.g., Lipetz, 1970).

The second stage of the wound response, i.e., traumatotaxis, has been reported in the vacuolated cells of several species during their response to wounding (e.g., Miehe, 1901; Pappelis et al., 1974; Ritter, 1911; Schnepf and von Traitteur, 1973; Tangl, 1884). It appears that such movement is accomplished by the nucleus moving through the cortical cytoplasm against the walls of the cell, rather than through the vacuole. Nuclear migrations in general have been examined—mainly using EM and drug studies—in several lower plants and fungi and in many of these organisms the movement appears to be mediated by microtubules. This is exemplified by the maintenance of a constant distance between the nucleus and the growing tip of the caulonema of the moss Funaria (Schmiedel and Schnepf, 1979a, b, 1980) and in the premitotic nuclear movement in spores of the fern Onoclea (Bassel et al., 1981)—see Britz (1979) and Gunning and Hardham (1982) for full reviews. In D. stramonium, microtubules, but no putative actin-filaments, were seen in sections using EM and a fibrillar cage around the nucleus was visualised by SEM. However, in the most thorough study of higher plant traumatotaxis using leaflets of the monocotyledon Tradescantia, microtubules did not appear to be involved. This conclusion was reached because nuclear migration was inhibited by cytochalasin B, but not by colchicine (Schnepf and von Traitteur, 1973). Furthermore, the inhibitors of mRNA synthesis, ethionine and actinomycin D, also prevent movement. Thus, it can be inferred that an actin-based system produced de novo, is responsible for the movement (Schnepf and Klump, 1975; Schnepf and Volkmann 1974). In regard to the role of mRNA in the current system, an increase in nucleolar volume was apparent in traumatotactic nuclei as was a swelling and rounding of the nucleus, both consistent with
an escalation of protein synthesis after wounding. Indeed, in potato tuber cells responding to wounding, an increase in nucleolar volume (Favali et al., 1984) and a rise in protein synthesis were observed (Borchert and McChesney, 1973). Prior to the first round of division in explants of Helianthus tuberosus, the nuclear surface enlarges almost immediately after cutting and nuclear volume increases five-fold, but only after a 4h lag (Williams and Jordan, 1980). However, in contrast to the present observations on D. stramonium, no change in nuclear pore density is apparent after wounding in H. tuberosus.

Cells with traumatotactic nuclei show an increase in cytoplasmic volume, compared with day 0 specimens, judged by the thickness of cortical cytoplasm seen using EM. This is more apparent at the level of the light microscope when, as first described by Sinnott and Bloch (1941a), the nucleus comes to reside in the centre of the cell in a cytoplasmic star. The arms comprising this systrophe contained microtubules, revealed by EM to run along the long axis of the strands. They also contained F-actin, as evidenced by their exhibiting streaming and by immunocytochemical studies (Chapter 5). Weidinger (1980a, b, 1982) has shown that formation of systrophes is an energy-requiring process and can be inhibited either by anti-microtubule or anti-actin drugs or by both, according to the species studied.

The majority of the strands then coalesce to form a phragmosome, as has been observed in other systems (e.g., Jones et al., 1960; Sinnott and Bloch, 1941a; Venverloo et al., 1980). However, there is no clear line of demarcation between the systrophic and phragmosomal stages. Phragmosomes often possess other strands (Sinnott and Bloch, 1941a, b; Venverloo et al., 1980; present study), although not invariably (Goosen-de Roo et al., 1984), and they may not be in the form of a complete diaphragm, but
instead possess tonoplast-lined perforations (Goosen-de Roo et al., 1984; Mahlberg et al., 1975). This is relevant in the context of attempts to discover whether the PPB or the phragmosome arises first during pre-mitotic preparations for division. In the current study, a cell with an obvious cytoplasmic star was observed with a PPB, as was a cell with a well-thickened phragmosome. If these cells are representative, PPB formation occurs prior to phragmosome development, but once the latter has formed the two co-exist. On the other hand, Venverloo et al. (1980) concluded that in Nautilocalyx explants, 'phragmosome formation was probably not preceded by the formation of a BMT' (band of microtubules, i.e., PPB), although, in a later conference report on the same system, this view was slightly modified (Venverloo and Pronk, 1982): 'These preliminary results suggest that the initiation of the BMT begins as early as the formation of the phragmosome'. However, in other publications from the same laboratory on Nautilocalyx and fusiform cambial cells of Praxinus, the relative timing of the two is not discussed further (Goosen-de Roo et al., 1980, 1984; Venverloo and Libbenga, 1981; Venverloo et al., 1984). Indeed, Goosen-de Roo et al. (1984) state that, 'the relation between the two above views [microtubules and phragmosomes controlling the division plane] is unclear'. As with the present study, in Venverloo et al.'s (1980) original report, only two cells were observed with a BMT. Furthermore, the cells used in their system form only an exceedingly thin (20-40nm) phragmosome in places. As a result the distinction between systrophe and phragmosome is even less marked than in D. stramonium. For example, the cell shown in Figure 9 of Venverloo et al. (1980), which possesses a BMT, has a rather stellar appearance. The authors also noted that, in cells that they considered to have not yet formed a phragmosome, some microtubules were observed in the expected plane and at the time of disappearance of the
nucleolus some still maintained microtubules at the BMT site. Thus, on the basis both of the data of Venverloo et al., and the results of the current study, it appears that a PPB forms after star formation, but prior to the completion of a phragmosome and persists until the early stages of mitosis. The PPBs observed in this study are more diffuse (around 10 µm in breadth) and contain fewer microtubules (maximum observed 40) than those regarded as typical of more dense meristematic cells (see Gunning and Hardham, 1982). This phenomenon of relatively less well-defined PPBs has also been observed in vacuolated cells undergoing 'programmed' divisions (Esau and Gill, 1969; Evert and Deshpande, 1970) as well as in those responding to wounding. Hardham and McCully (1982a) found that the PPBs in regenerating pea roots contained between 29 and 45 microtubules, with the most defined containing 37 microtubules, covering a distance of 2.5 µm, although some microtubules were found elsewhere in the cell cortex. Venverloo et al. (1980) noted BMTs possessing 30-40 microtubules in bands 2-4 µm wide (50 microtubules in 3-6.4 µm given in Venverloo and Pronk, 1982) with, as in the current study, no microtubules present in the remainder of the cortical cytoplasm. The less diffuse nature of the PPBs in these reports, compared with that of D. stramonium pith cells, may reflect the smaller volume of the cells of Nautilocalyx (approximately 10 times smaller) and pea roots (more than 25 times smaller).

The low number of microtubules in PPBs of vacuolated cells has been associated with the few cortical microtubules seen in such cells, reflecting the possible utilisation of a common pool of tubulin (see Hardham and McCully, 1982a). However, this does not appear to apply to the current system for, although not prominent in terms of density, many microtubules are seen in the cortex associated with the wound-induced deposition (Chapter 4). Thus, if a common pool of tubulin is utilised, or
PPBs form as a result of coalescence of cortical microtubules (Wick and Duniec, 1983), it may have been expected that more of them would be found in the PPBs. Perhaps the PPBs observed did not represent the maximum density attained. Unless large, transient PPBs do develop, it would appear that the PPB can function effectively with fewer microtubules in vacuolated cells than it can in cells which are smaller and contain little or no vacuole.

Microtubules were also observed in the phragmosome between the PPB and the nucleus, many of them associated with the nuclear envelope and lying parallel with the PPB, although others were seen in different orientations. Using immunofluorescence on onion roots, Wick and Duniec (1983, 1984) observed perinuclear microtubules which were parallel with the PPB or randomly directed; spindle microtubules formed at a later stage. In Sphagnum Schnepf (1984), using EM, reported an equivalent transition state between the PPB and spindle, where the microtubules were also parallel with the former. A similar phenomenon can also be seen in Figure 3 of Cronshaw and Esau (1968) in a study of cell division in Nicotiana leaves. It seems unlikely that such microtubules represent those of the early spindle as the majority of them are aligned at 90° to the spindle's future axis. The microtubules could represent the polymerisation of free tubulin around the nucleus, as has been suggested might occur by Wick and Duniec (1983, 1984). However, Goosen-de Roo et al. (1984) have observed microtubules aligned along the length of phragmosomes from before the onset of mitosis to the stage of cell plate formation. These authors suggest that these microtubules are representative of a further category of microtubules which guide the centrifugally-growing cell plate to the parental wall and that they may also occur in non-vacuolated cells. Whilst microtubules were observed in the radiating cytoplasmic arms of systrophes and between nuclei
and PPBs, they were not seen in the phragmosomes beyond the margin of the phragmoplast in the current study.

Phragmoplast and cell-plate formation in *D. stramonium* pith cells follows the typical pattern of that of walled higher-plant cells, extending centrifugally and apparently guided by the phragmosome. This has been observed in other vacuolated cells (e.g., Goosen-de Roo *et al.*, 1984; Sinnott and Bloch, 1941a; Venverloo *et al.*, 1980). Paradoxically, it appears that, as noted by Wada (1950), cell plate maturation takes place centripetally. This is illustrated by the image (Fig. 2.68a) of a cell plate which is fairly mature on one side where it has fused with the parental wall, less mature in the centre and still possessing phragmoplast microtubules at the opposite side. Also apparent is the ER which is parallel with and penetrates through the cell plate. This was studied in detail by Hepler (1982). It provides further support for the concept that the desmotubule of plasmodesmata is formed from ER (see Overall *et al.*, 1982a, b).

It has been suggested that phragmosomes, the other early manifestations of the division plane in vacuolated cells, occur in non-vacuolated cells, but are not normally observable (see Gunning, 1982a). For example, in a classic study, Öta (1961) centrifuged densely-cytoplasmic *Tradescantia* stamen hair cells and found that a raft of cytoplasm remained across the vacuole that had been formed, this artificially-isolated phragmosome predicting the line of the future cell plate. From this result it might be anticipated that the phragmosomal cytoplasm would display cytological specialisation. However, in untreated cells of the same type, differences between the cytoplasm in the area of the future cell plate and that in the rest of the cell are not readily discerned, either in the distribution of organelles (other than the PPB microtubules) (Busby and
Gunning, 1980) or as gross differences in cytoplasmic density. Observations made in conjunction with the work on *D. stramonium*, but not included in this thesis, showed no detectable differences in optical path difference (OPD) of the cytoplasm during pre-prophase in *Tradescantia* using uniform field interference optics (see Section 3.2.4), although the system used was sensitive down to changes in OPD of less than 1/100th of a wavelength (Beyer, 1974). It was able to show, for example, that organelles have a different OPD from the cytoplasmic strands along which they are moving. Furthermore, in *D. stramonium*, as also noted for *Nautilocalyx* by Venverloo et al. (1980), the cytoplasm of the phragmosome and the cell cortex appeared to be the same in their electron microscopic images. Clearly, differences between the phragmosome and the remaining cytoplasm are subtle and have still to be characterised.

Systrophic strands, phragmosomes and subsequent cell divisions in adjacent cells of *D. stramonium* pith often line up in 4-way junctions. This feature of wound-induced divisions (Sinnott and Bloch, 1941b) is normally avoided in plant development, although it does sometimes occur, for example, in some of the programmed divisions in the *Azolla* root (Gunning et al., 1978a) and also in normal periderm development (see Sinnott and Bloch, 1941b). Another characteristic of initial wound-induced divisions in many species is that the overwhelming majority of them are parallel with the cut surface (Gunning, 1982a; Sinnott and Bloch, 1941b). This was certainly the case in the responding *D. stramonium* pith cells. Quantitative analysis of transverse sections of the same type of wound in tomato stems, revealed that nearly 70% of the first new walls were at an angle of 10° or less to the cut surface while less than 10% were at an angle of 50° or more (Warren Wilson and Grange, 1984).

The mechanisms that are involved in the organisation of wound-induced
divisions are of considerable interest, as they also may be factors in the control of plant development in general. In the wound response of *D. stramonium*, it can be shown that the traumatotactic nuclear migration is associated with the change in cell polarity, but not with the organisation of the division plane. In transverse wounding experiments (see Section 4.3.6), divisions (also often lined up in 4-way junctions) occur parallel with the wound surface, but there is no change in cell polarity and no traumatotaxis occurs. All of the other events observed (at the level of the light microscope) in the case of longitudinal wounding take place. Both in the present system and others, it has been proposed that subsequent tissue differentiation is controlled through the agency of hormones (Warren Wilson, 1978). The 'gradient induction hypothesis', which has considerable experimental support (Warren Wilson, 1982; Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1961a, b, 1963, 1981, 1983), suggests that opposed gradients of auxin and sucrose control the position of cambium formation. Although the cambium does not appear as such until around day 10, it has been shown that its position has been determined by the time of the first round of division (Evans, 1979; Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1983). It also seems likely that the traumatotactic nuclear movement is in response to a chemical gradient, as indicated, for example by the inhibition of traumatotaxis in *Tradescantia* by the washing of the wound surface (Schnepf and Volkmann, 1974). However, an auxin gradient is thought not to be responsible in the same species (Schnepf and von Traiteur, 1973). It has been suggested that oligosaccharides released from plant cell wall pectic polysaccharides at a wound may play a hormone-like role in regulating plant responses remote from the site of release (Bishop *et al.*, 1981; McNeil *et al.*, 1984; Ryan *et al.*, 1981).
However, as mentioned by Sinnott and Bloch (1941b) and discussed by Gunning (1982a), it is not obvious how a gradient across a cell can control the division plane to the degree of precision that is apparent. With regard to a possible chemical signal it is noteworthy that the initial stimulus, as manifested by organelle enclustering of the nucleus and its subsequent migration, traverses a large number of cells, while subsequent divisions occur only in a relatively shallow layer. Thus, there is a parallel with wound healing in potato tubers, where DNA synthesis is confined to the first 2 or 3 cell layers below the wound where cell divisions and starch degradation are observed later, while increased protein synthesis and respiration occur much deeper into the wound (Borchert and McChesney, 1973). Using the criterion of the appearance of mitotic figures as a sign of activation after wounding in stems of three dicotyledonous species, including *D. stramonium*, Warren Wilson and Grange (1984) noted that it was uncertain why, if diffusion of a morphogen was responsible, activity was restricted to a shallow layer under the wound surface. The observation that traumatotaxis reaches fairly deeply into the sub-wound tissue, seems to resolve this apparent contradiction of restricted diffusion.

It can be argued that mechanical stress is a more likely influence than hormonal régimes in the determining of the plane of division, at least in the current system. It was pointed out over 80 years ago (Kny, 1902; Němec, 1899) that wounding leads to local alterations in the pattern of stress and the resultant changes in internal tensions could be responsible for orienting the early divisions during wound responses. In more recent history, stress (as pressure) was shown to be an important factor in wound reactions by Brown and Sax (1962). They discovered that, if a longitudinal strip of bark was removed from the bole wood of *Populus* and *Pinus*, wound-
induced divisions occurred leading to the reformation of the lost tissue. However, if the bark, wrapped in plastic, was reapplied and firmly held in place, the stem behaved normally. In a later series of papers Lintilhac and co-workers (Lintilhac, 1974a, b; Lintilhac and Jensen, 1974; Lintilhac and Vesecky, 1980, 1981, 1984) have shown that the final orientation of the cell plate is in a position maximally protected from shear, i.e., perpendicular to the largest non-zero principal stress passing through the cell; a conclusion similar to that reached by the first worker on the effects of stress upon the plane of cell division (Kny, 1896, 1902).

The internal tissue of an intact stem is under compression, as shown by the observation that each half of a split stem bows outward (see Lang et al., 1982; Taiz, 1984). The surgery carried out on the D. stramonium stem would, therefore, be expected to result principally in radially-oriented stresses in the area under the cut surface. Indeed, the wound-induced divisions are generally at 90° to the radial direction and are very similar in appearance to the co-planar divisions seen in pith explants of Nicotiana when an external compressive force is applied (see Fig. 1c of Lintilhac and Vesecky, 1984, and the effects of stress on division in Coleus internodes, Lintilhac and Vesecky, 1981).

However, as pointed out by Gunning (1982a), it is apparent that, if stress relationships are important, they must manifest themselves earlier than cell plate formation as, by that stage, the division site has already been determined. Indeed, Lintilhac (1974b) suggested that the PPB may be associated with the shear-sensitive step. In the D. stramonium pith cell response, phragmosomes predict division planes, but these appear to be preceded by PPBs (this is discussed above) which in turn are thought not to be the initial determinants of the plane of division (e.g., Pickett-Heaps, 1969a, b). One must, therefore, look for an earlier structure, perhaps
connected with the nucleus. In an elegant study on *Onoclea*, Miller (1980) concluded that, 'the primary factor which determines the plane of division...is the local pattern of stress which exists at the position of the nucleus at the time of onset of cell division and wall formation'.

In the *D. stramonium* system, the key stage could be that in which the nucleus is positioned in the centre of the cell in a systrophe. This organisation could be a shear-sensitive cytoplasmic structure, capable of perceiving minute distortions at the cell surface and somehow transducing them in order to establish the division site. The radiating strands of cytoplasm would appear to be suitably positioned to act as multidirectional stress receptors, whether directly or through the agency of components within them. Microtubules could play this role as they are capable of originating shearing movements (Tucker, 1972) and therefore could be expected to be sensitive to shear (Lintilhac, 1974b). In this regard, microtubules have been observed associated with nuclear extensions in the prelude to division in vacuolated cells of *H. tuberosus* explants (Yeoman et al., 1970). These extensions were observed only from the appearance of trans-vacuolar strands, through phragmosome formation, until the onset of nuclear envelope breakdown. Yeoman et al. (1970) considered the extensions and associated microtubules to be involved in the premitotic nuclear movement, but perhaps they could also function as the termini of stress receptors. However, Lloyd and Barlow (1982) reject microtubules as the likely cytoplasmic stress receptors on the grounds that they are too dynamic. They propose that the system of fibrils - which do not appear to be composed of tubulin or actomyosin (Lloyd et al., 1982) - which has been shown to connect the nucleus to the cell periphery in suspension culture from four plant species, is a more plausible candidate. The authors relate this structure in plants to the nucleus-
anchoring system of intermediate filaments found in animal cells (Lehto et al., 1978). The fibres described by Lloyd et al., (1982) may also occur in other types of cell. Blackmore et al., (1984) have demonstrated, using SEM of frozen specimens, that a system of fibrils, considered by the authors to be microtubules not preserved by normal fixation procedures, emanates from nuclei in cells of leaves of Aucuba.

Whatever the nature of the receptors and transducers, it could be that the radiating arms of the systrophe act in a manner similar to that of the supporting strands of a spider's web, positioning the nucleus and division site in the (mechanically) most stable position. In this regard, it is noteworthy that in hand-sectioning experiments (see Section 5.3.1) decollation of cells at most stages of the wound response generally left the cytoplasm relatively intact. However, such truncations always caused the destruction of systrophes thus demonstrating their delicate nature. Indeed, to extend the above analogy further, when a single guy of a spider's web is severed, this usually causes the web to collapse. Strands traversing vacoulated cells are not static and move their anchoring position through the cortical cytoplasm (Kirschner and Sachs, 1978; Venverloo et al., 1980). It may be that the strands move until they come to reside along the line of least stress. Once established, more strands gradually come into the same plane until a phragmosome is formed.

The above view may be supported by the observation in the present system that the arms of adjacent systrophes often, as phragmosomes subsequently do, line up in 4-way junctions. Also, Venverloo et al. (1980) noted that some of the pre-phragmosomal strands also predicted the subsequent position of the new wall. Lloyd (1984b) stated that, 'the phragmosome can lay claim to the title of being the organelle least understood yet most likely to help our understanding of pattern formation -
the task now being to find out more about its structure and about the forces that orientate it'. It may be that a system of cytoskeletal fibrils, not usually preserved or difficult to visualise or both, occurs in the arms of systrophes in vacuolated and non-vacuolated cells, and are the components involved in initial positioning of the phragmosome.

That changes do occur in response to wounding, in some structural element(s) of the cytoplasm, is indicated by the work of Sachs (1972). He carried out slow, but severe, plasmolysis of hand sections of hypocotyls of *Helianthus annuus* and of other plants and showed that in mature tissue the shrunken protoplast always lay at right angles to the major direction of growth and parallel with the division plane, i.e., transverse. Furthermore, upon longitudinal wounding, the pattern changed after 24h to become longitudinal in cells in the vicinity of the wound, thus predicting the change in polarity that was later manifested by divisions parallel to the wound. Sinnott and Bloch (1941a) also noted that the edge of the phragmosome was the portion of the cytoplasm most firmly adherent to the wall in plasmolyzed cells. This may be related to the observation of a reticulate band of cytoplasm at the outer edge of the phragmosome in live cells (Roberts and Northcote, 1970; Venverloo *et al.*, 1980).

With respect to any hypothesis concerning the role of stress in the orientation of the division plane in responding *D. stramonium* pith cells, it should be borne in mind that a considerable amount of wall thickening occurs, not only prior to division, but before the formation of systrophes (see Chapter 4) and that this may change the stress patterns through the tissue. In this regard, Lintilhac and Vesecky (1981) observed an acceleration of secondary wall deposition in maturing xylem elements of *Coleus* when the stem was subjected to an applied mechanical stress. However, in experiments on explants of *H. tuberosus* Yeoman and Brown (1971)
noted that, 'Stress as a result of bending is effective only during the first 24h. In spite of this it continues to exert an effect over 7 days'.

Once the change in polarity (as manifested by traumatotaxis) has been initiated, it is maintained - as nuclear migration to the wound wall does not occur in subsequent divisions. However, all the other phenomena associated with the initial division do occur, including the overall temporal sequence from systrophes to phragmosomes to mitotic figures. However, fewer divisions line up at 4-way junctions. This alteration in alignment of new walls was also observed by Sinnott and Bloch (1941b). They noted that 4-way junctions tended to occur in the thicker areas of wall. Where the wall was thinned by expansion, staggered junctions predominated. This also appears to be so in *D. stramonium* where, as also described by Sinnott and Bloch (1941b), novel intercellular spaces develop at 4-way junctions, distinguishable in transverse sections by their diamond shape as compared with the generally triangular outline of the pre-existing ones.

An increasing number of anticlinal and oblique divisions occur in the subsequent rounds of divisions. These more than overcome the effects of radial expansion and cell size decreases markedly in all dimensions. This is thought to be a requirement for cambium formation at a later stage (Warren Wilson and Grange, 1984).

In the wound response of *D. stramonium* (and other species) Warren Wilson and Grange (1984) have divided the regeneration process into lag (prior to the initial division), division (from the initial divisions until tissue differentiation) and differentiation (appearance of cambia and associated events) phases. The authors stress that such a partition is not rigorous and that the transitions between phases are not instantaneous. The work in the current study focusses on the lag phase, where the
considerable protoplasmic reorganisation described above occurs, and the early part of the division phase (which ends around days 9 or 10, Evans 1979; Warren Wilson and Grange, 1984). The cells involved in the wound response are usually described as being differentiated prior to wounding, de-differentiated during the division phase and (re-)differentiated during the reformation of the tissue into easily-ascrivable types (e.g., Bloch, 1941; Warren Wilson and Warren Wilson, 1983). Implicit in the term 'de-differentiation' is a lack of organisation, especially in its use to describe amorphous masses of dividing cells in tissue culture systems (e.g., see articles in Street, 1977). However, it is apparent that in response to wounding, D. stramonium pith cells, prior to the initial division, undergo an organised protoplasmic change and a controlled deposition of wall material (see Chapter 4) in a strikingly-ordered fashion, for which the term de-differentiation seems quite inappropriate. These cells would be better categorised as re-differentiating directly, without division, into a new cell type which, although not immediately definable in conventional terms, is recognisable, ordered and becomes meristematic. In the light of these observations the lag phase could also be termed the primary re-differentiation phase.

Subsequent chapters in this thesis examine further aspects of the primary re-differentiation phase, especially those aspects related to cell wall deposition. The foundation is given in Chapter 3, which describes the pre-wounding state of the wall. Chapter 4 then analyses post-wounding wall developments. Chapter 5 returns to the cytoplasm and details preliminary attempts to investigate the roles of cytoskeletal microtubules and actin filaments using immunofluorescence microscopy.
FIGURE LEGENDS - CHAPTER TWO

A list of abbreviations used in all figure legends in this thesis is given on page vi.

In images of longitudinal sections the double-headed arrow indicates the approximate long axis of the stem.

Figures 2.1 - 2.2:

Surface views of the wound.

Figure 2.1:

Photographs of a Datura stramonium plant before- (a) and immediately after- (b) wounding. Starting at the petiole of the last leaf (asterisk) below the crotch (arrowhead) a vertical slab of stem, approximately one third of its width and 2cm in length, is removed. (a) x 0.5 approximately, (b) x 1 approximately.

Figure 2.2:

Surface views of the wound response in a single specimen. (a) before wounding; (b) 0, (c) 2, (d) 3, (e) 4, (f) 5, (g) 6, (h) 7, (i) 8, (j) 9, (k) 10, (l) 11, (m) 12, (n) 14, (o) 16, (p) 18, (q) 20, (r) 25 days after wounding.

It is apparent that the length of the wound does not increase during healing. However, soon after division start (around days 3 (d) or 4 (e)), a considerable radial expansion takes place which results in the wound becoming markedly wider than the tissue above and below it (e.g., (h)). Later, the difference in girth of the wound compared with the adjacent stem declines ((i) - (r)).

During the early stage of the response the outer cortical (arrow), vascular (arrowhead) and pith (asterisk) tissue can be clearly seen. The vascular tissue is initially pushed outward by the divisions in the pith (e.g., (g)), but later, after the vascular and cork cambia have presumably formed, the surface of the wound smooths out ((k) - (r)).
Figures 2.3 - 2.9:

Pith cell anatomy before wounding.

Figure 2.3:

Transverse SM-section illustrating the highly-vacuolated polygonal-nature of the cells. Cytoplasm is only apparent where amyloplasts (e.g., arrowhead) occur. Triangular intercellular spaces (e.g., I) are found at cell junctions, where wall thickening is also evident (e.g., arrows). Köhler illumination x 215.

Figure 2.4:

Transverse hand-section; as Figure 2.3 except that the flattened nuclei (e.g., arrow) can also be seen. I, intercellular space. DIC optics x 215.

Figure 2.5:

Transverse section as seen using SEM, revealing the polyhedral nature of the cells with abrupt side/side (black arrowhead) and end/side (white arrowhead) boundaries within the cells. Flattened nuclei (arrows) can be seen lying against side or end walls. x 400.

Figure 2.6:

Longitudinal SM-section showing the elongated shape of the cells, with longitudinally-running intercellular spaces (e.g., I). The majority of end walls (e.g., E) are transverse, but some are oblique (arrow). Amyloplasts (e.g., arrowhead) mainly lie by basal end walls. Köhler illumination x 215.

Figure 2.7:

Longitudinal hand-section showing the longitudinal continuity of the intercellular spaces (I). Flattened nuclei (e.g., arrow) and the oval pits of the side wall (e.g., arrowhead) can also be seen. DIC optics x 215.

Figure 2.8:

Transverse SM sections of a crystal-containing cell examined using Köhler illumination.

(a) Stained with both toluidine blue and the Pizzolato silver-stain for calcium oxalate (see Section 2.2.3.2). The crystals (asterisk) are darkly stained.

(b) Serial section to (a) stained with toluidine blue only. The crystals (asterisk) are unstained. (a) and (b) x 900.

Figure 2.9:

Electron micrograph of a transverse section showing a typical elongated, flattened, nucleus (N) lying against a longitudinal wall (star), which is transversely reinforced with cellulose (see Chapter 3). Nuclear pores (e.g., arrows) can also be seen. x 9,000.
Figures 2.10 to 2.18:

Figure 2.10 - 2.12: Pith cell anatomy before wounding.

Figure 2.10:

Electron micrograph of a transverse section showing a crystal-containing cell. As with other cells, the cytoplasm is restricted to a thin veneer lining the walls (arrowhead). In this case, however, the vacuole is littered with crystals, the sites of which can be judged by the irregularly-shaped holes (e.g., asterisks) that have been left as a result of crystal removal by the knife during sectioning. x 4,000.

Figure 2.11:

Electron micrograph of a longitudinal section showing links (e.g., arrowheads) between transversely-oriented cortical microtubules (arrows) and the plasma membrane (star). x 150,000.

Figure 2.12:

Electron micrograph of the cytoplasm underlying an end wall as seen in transverse section. Putative MAPs (e.g., arrowheads) are present, linking adjacent microtubules. x 50,000.

Figures 2.13 - 2.18:

Transverse SM sections of the wound examined using Köhler illumination. All figures are oriented as if the wound was up and down the left-hand side of the page. Ep, epidermis; Co, collenchyma; Oc, outer cortex; Ph, phloem; Xy, xylem; Pi, pith.

Figures 2.13 - 2.15:

Days 1-3 respectively. As observed in surface views of the wound response (Fig. 2.2), little change is apparent. All x 18.

Figure 2.16:

Day 4. The first round of division has occurred in many of the pith cells underlying the wound (the new walls are not readily visible at this magnification) especially near the vascular tissue (site marked by arrowhead). x 18.

Figure 2.17:

Day 5. Two or more rounds of division and some radial expansion have taken place in the sub-wound pith cells. Many divisions having occurred near the vascular tissue (arrowhead). A moribund tier one to two cells deep is visible (arrow) immediately under the cut surface. It is apparent that the SM binds to the surface of the wound (star) but not to the epidermal cuticle (asterisk). x 18.

Figure 2.18:

Day 6. Several rounds of division and marked radial expansion has occurred in the sub-wound pith cells. A zone of moribund cells much deeper than was normally observed is present under the cut surface (arrow). Some divisions have also taken place in the outer cortical cells (arrowhead), but little or no meristematic activity occurs in the vicinity of the wound in the epidermis, collenchyma or mature vascular tissue x 14.
Figures 2.19 - 2.26:

The cytoplasmic wound-response as visualised in hand sections using DIC optics. All figures, except Fig. 2.25 where the wound would have been across the top of the page, are positioned as if the wound was up and down the left-hand side of the plate.

Figure 2.19:

Unstained transverse-section, 8h after wounding, showing several organelle-enclustered nuclei (arrowheads) at various positions on the cells' periphery. x 215.

Figure 2.20:

Day 1; stained transverse-section. Cytoplasmic strands can be seen radiating from a traumatotactic nucleus (black arrowhead). A systrophe (arrow) and an organelle-enclustered nucleus (white arrowhead) are also present. x 215.

Figure 2.21:

Day 2; stained longitudinal section showing systrophes near- (white arrows) and pulled away from- (black arrow) the wound wall. An unmoved nucleus (arrowhead) is also present in a cell that is immediately under the cut surface (not shown). x 270.

Figure 2.22:

Day 2; stained transverse section. Higher magnification view of a systrophe showing the fine, radiating cytoplasmic-strands (e.g., arrows) and the nucleus enclustered by organelles (arrowhead). A traumatotactic nucleus (star) is visible in an adjacent cell. x 540.

Figure 2.23:

Day 3; stained transverse section. The first round of division has occurred, in many cells, parallel to the wound and with adjacent divisions often abutting each other exactly (e.g., arrowheads). This lining-up in adjacent cells is also sometimes observed with systrophic strands (arrows). x 215.

Figure 2.24:

Day 4; stained transverse section. Several divisions have taken place, most of them parallel with the wound. In these later divisions, many of the new cross-walls, as in the initial divisions, abut each other exactly (e.g., arrows). A degree of radial expansion has occurred, but the mother-cell walls are still readily distinguishable (e.g., arrowheads). x 215.

Figure 2.25:

Day 3; transverse hand-section. Divisions have occurred parallel to the wound. A phragmosome (arrowhead) and a systrophe (arrow) are also present. x 215.

Figure 2.26:

Day 4; longitudinal hand-section. In the later rounds of division, an increasing number of new walls occur which are oriented other than periclinally (e.g., arrows). x 215.
Figures 2.27 - 2.28:

SEM of transverse sections from wounded tissue. All figures are oriented as if the wound was up and down the left-hand side of the page.

Figure 2.27: Day 3

(a) View, all of the early stages of the cytoplasmic wound-response can be seen. Immediately under the cut surface there are collapsed cells (star); further away an organelle-enclustered- (black arrow), traummatotactic-(white arrowhead) and systrophic- (black arrowhead) nucleus are present. x 185.

(b) Close-up of the cell from (a) that contains the organelle-enclustered nucleus. Fibrils (e.g., arrows) are seen attached to some of the enclustering organelles. x 780.

(c) Detail of the traumatotactic nucleus seen in (a). A network of filaments can be seen (e.g., arrows) that envelopes the nucleus (N) and its associated organelles. x 1,600.

(d) Higher magnification view of the systrophe-containing cell from (a). The systrophe is still near the wound wall and has yet to reach the centre of the cell. The radiating cytoplasmic strands can be clearly seen (e.g., arrows), although some appear to have broken during preparation. The fairly abrupt side/side wall edges (e.g., arrowhead) are also apparent. x 450.

Figure 2.28:

Vista of the pith cells in a day 5 wound. Several rounds of division have taken place, the majority of which are parallel with the wound, although anticinal and oblique walls are also apparent. Some divisions exactly line up in adjacent cells (e.g., black arrow), whilst others do not (e.g., white arrow). Considerable radial expansion has taken place in the meristematic cells, but the outlines of the mother-cell walls can still be traced in many cases. From these it can be seen that divisions only occur in a tier of cells 3 to 4 deep that lie beneath the non-responding cells, which are present immediately under the cut surface (e.g., stars). x 65.
Figures 2.29 to 2.35:

The early stages of the wound response as visualised in SM sections. All figures are oriented as if the wound was up and down the left-hand side of the page.

Figure 2.29:

Day 1; transverse section; low magnification view showing several traumatotactic nuclei (e.g., arrows). Cells immediately under the wound have collapsed (e.g., star) or appear likely to do so, although in some of the latter the initial response may have occurred (arrowheads). Köhler illumination. x 185.

Figure 2.30:

Day 3; transverse section. No divisions have yet occurred, but some wall thickening is apparent in responding cells (e.g., asterisk) compared with the collapsed walls (e.g., star) immediately under the wound. Köhler illumination. x 185.

Figure 2.31:

Day 4; transverse section. As Figure 2.30 except that wall thickening (e.g., asterisk) is more apparent and the first round of division has taken place, parallel with the wound, in some cells (e.g., arrows). Köhler illumination. x 185.

Figure 2.32:

Day 1; transverse section. Close-up of a traumatotactic nucleus lying against the wound wall. The refractile granules (e.g., arrowheads) are amyloplasts. The nucleolus (arrow) can also be seen. A-DIC optics. x 540.

Figure 2.33:

Day 3; transverse section showing a systrophe not yet in the centre of the cell. Clustered organelles (arrowhead) mask the nucleus. Fine cytoplasmic strands (e.g., arrow) and wall thickening (asterisk) are also apparent. DIC optics. x 540.

Figure 2.34:

Day 1; longitudinal section. As Figure 2.33, except that the position of the nuclei half-way up the wound wall is manifested. I, intercellular space. A-DIC optics. x 540.

Figure 2.35:

Day 4; transverse section. A systrophe, recent division and a traumatotactic nucleus can be seen. All nuclei possess nucleoli (arrows). Wall thickening (e.g., asterisk) is also apparent. A-DIC optics. x 540.
Figures 2.36 - 2.43:

Mitotic events, as visualised in the later rounds of division. All figures are oriented, except Figure 2.40 where it would have been across the top of the plate, as if the wound was up and down the left-hand side of the page.

Figure 2.36

Day 4; stained hand-section. After the first division has been completed, the pre-mitotic cytoplasmic events, apart from traumatotaxis, are repeated. In this image, cytoplasmic strands (e.g., arrow) traversing the vacuole to a nucleus by a recent division can be seen. This is the first stage in the formation of systrophes. DIC optics. x 215.

Figure 2.37:

Day 7; transverse SM-section. Several divisions have taken place, the majority parallel with the wound. A phragosome (star) and nucleus with a trans-vacuolar strand (arrow) can be seen. Also present is a novel diamond-shaped intercellular space (asterisk) which has apparently formed at the site of a 4-way junction. A-DIC optics. x 540.

Figure 2.28:

Day 6; transverse SM-section. Three systrophes/phragmosomes (arrows), two of which have chromosomes visible, are present in the daughter cells of a pith cell, which has undergone two rounds of periclinal divisions (arrowheads). Examination of serial sections did not permit the stages of mitosis to be identified. A-DIC optics. x 540.

Figure 2.39:

Day 6; transverse SM-section showing a metaphase plate (arrow) in a phragmosome. A-DIC optics. x 675.

Figure 2.40:

Day 6; transverse SM-section showing a mitosis at late anaphase in a phragmosome. A-DIC optics. x 830.

Figure 2.41:

Day 6; transverse SM-section. A cell plate can be seen (arrow) forming in a phragmosome parallel to the wound (seen at lower magnification in Figure 2.47). A-DIC optics. x 540.

Figure 2.42:

Day 7; transverse hand-section. A cell plate (arrow), creating a transverse division, is seen en face. DIC optics. x 830.

Figure 2.43:

Day 7; transverse hand-section. A cell plate (arrow), which is creating an anticlinal division, can be seen end on. In this case, the phragmosome (star) plus other supporting strands (e.g., arrowhead) are apparent across the whole diameter of the cell. DIC optics. x 830.
Figures 2.44 - 2.48:

SM sections of the wound, examined using Köhler illumination, after several rounds of division have taken place. All figures are oriented, except Figure 2.40 where it would have been across the top of the plate, as if the wound was up and down the left-hand side of the page.

Figure 2.44:

Day 7; transverse section. Many periclinal as well as anticlinal and oblique divisions have markedly-reduced average cell size. Collapsed (e.g., star) and non-responded (e.g., asterisk) cells occur under the cut surface. Considerable radial expansion has taken place, but the mother-cell walls (e.g., arrowheads) are still recognisable in some cases. Novel, diamond-shaped intercellular spaces (e.g., arrow) have formed at the site of some 4-way junctions. x 65.

Figure 2.45:

Day 7; as Figure 2.44, except a longitudinal section. x 65.

Figure 2.46:

Higher magnification image of a portion of Figure 2.44 showing crystals (e.g., arrows) in several daughter cells. x 270.

Figure 2.47:

Day 6, transverse section. The radial expansion that has occurred in the mother-cell walls (e.g., arrowheads) is most apparent when comparing their shape with that of the non-responded, but non-collapsed, cells (e.g., asterisk) immediately under those which have collapsed. The cell-plate containing cells is shown at higher magnification in Figure 2.41. x 185.

Figure 2.48:

Day 5; transverse section. Mother-cell walls are again visible (e.g., arrowheads), as is the radial expansion that has taken place in the responding, but not in the moribund (e.g., asterisk), cells. This example is somewhat atypical in that the first round of division has taken place anticlinally (arrows). I, intercellular space. x 185.
Figures 2.49 - 2.52:

Electron micrographs.

Figure 2.49:

Day 1; transverse section showing microtubules (arrows) near the nucleus. X 30,000.

Figure 2.50:

Day 1; longitudinal section. The nucleus, containing a large nucleolus (asterisk), is much more rounded and has a greater frequency of nuclear pores (e.g., arrows) than before wounding (cf. Figure 2.9). Also apparent are an enclustering amyloplast (star) and post-wounding deposition (arrowhead) in the outer periclinal wall (see Chapter 3). X 8,000.

Figure 2.51:

Day 1; periclinal longitudinal section. Traumatotactic nucleus as seen en face (the wound would have been positioned parallel with and above the page). Enclustering organelles (stars), microtubules (e.g., arrowheads) and grazing views of nuclear pores (e.g., arrows) are visible. X 17,000.

Figure 2.52

Day 4; transverse section (the wound would have been across the top of the page). Two adjacent new cells (asterisks) meet at a 4-way junction. A novel intercellular space appears to be starting to form (star). X 27,000.
Figures 2.53 - 2.57:

Day 5 pith cell, which possesses a PPB, as seen in electron micrographs of transverse sections. Adjacent cells have divided periclinally (not shown).

**Figure 2.53:**

Low magnification vista; the wound was roughly parallel with the outer periclinal (OP) wall. A phragmosome (stars) containing the nucleus (N) lies across the cell between the radial (R) walls parallel to the wound. Other supporting cytoplasmic strands (e.g., arrowheads) are also present. x 5,000.

**Figure 2.54:**

Higher magnification image of the supporting strand marked with an arrowhead in Figure 2.53. Microtubules (e.g., arrows) are visible near the nucleus, but not in the strand itself or in the cytoplasm underlying the wall. The same distribution was observed in over 20 serial sections (not illustrated). x 30,000.

**Figure 2.55:**

The base of the lower portion of the phragmosome seen in Figure 2.53. Several transversely-sectioned, and therefore longitudinally-aligned, cortical microtubules are apparent (e.g., arrows). In addition, some microtubules (e.g., arrowheads) are seen in the phragmosome. x 40,000.

**Figure 2.56:**

Grazing view of the nucleus (N) from a serial section, in the same orientation as Figures 2.55 and 2.57. Microtubules (e.g., arrowheads) are seen near the nuclear envelope and associated with nuclear pores (arrow). The majority of the microtubules are aligned parallel with the phragmosome and, therefore, the PPB. x 30,000.

**Figure 2.57:**

The base of the upper portion of the phragmosome labelled in Figure 2.53 as seen in a serial section. Transversely-sectioned cortical microtubules (e.g., arrows) are seen. These and those visualised in Figure 2.55 are presumably part of an encircling, periclinally-aligned, PPB. Also present, amongst the PPB microtubules, is a transversely-oriented (arrowhead). It is unclear whether this is associated with the PPB microtubules or those that populate the phragmosome. x 40,000.
Figures 2.58 - 2.64:

Systrophic day 4 cell, which also contains a PPB, as seen in longitudinal section. The figures are positioned so that the wound would have been approximately up and down the left-hand side of the page. All images, except Figure 2.59, are electron micrographs.

Figure 2.58:

View of the nucleus in a systrophe. The PPB lies at the base of the strands marked with arrowheads. The capitals of several other of the columns of cytoplasm that comprise the systrophe are also visible. As an aid to determining the relative orientations of different figures, the asterisked features are arrowed in Figure 2.59. x 6,000.

Figure 2.59:

The systrophe from Figure 2.58 as seen using A-DIC optics in a serial section, approximately 2 μm thick and stained with toluidine blue. The mother cell has divided once parallel to the wound (asterisk), as have its neighbours. One daughter cell is undergoing cell plate formation (arrowhead) and the other contains the systrophe and PPB, both of which are also aligned parallel to the wound. Several anchoring points (a-f) are present in the cell cortex of the systrophic cell. Of these only (a) and (f) have cortical microtubules at their bases and thus represent portions of an encircling PPB. The arrowed features are marked with an asterisk in Fig. 2.58. x 400.

Figure 2.60:

The base of strand (d). No cortical microtubules are present. This was also the case in serial sections of both this strand and anchoring points (b), (c) and (e) (not illustrated). x 35,000.

Figure 2.61:

The base of strand (a) showing the PPB of transversely-sectioned cortical microtubules (e.g., arrows). As this is from a longitudinal section the wall is an end wall and so the PPB is in the same orientation as that illustrated in Figures 2.53 - 2.57. x 45,000.

Figure 2.62:

The base of strand (f) showing the other portion of the PPB of cortical microtubules (e.g., arrows). x 45,000.

Figure 2.63:

A higher magnification view of a portion of Figure 2.58 showing microtubules (e.g., arrows) near the nucleus (N). As noted in Figure 2.56, the majority of the microtubules are aligned approximately parallel to the PPB, although in this example they are not as associated with the nuclear envelope to such an extent. x 20,000.

Figure 2.64:

A portion of strand (d) showing a microtubule (arrow). The occasional microtubule was seen in the strands even when they lacked microtubules at their bases. x 30,000.
Figures 2.65 - 2.67:

Spindle and phragmoplast microtubules.

Figure 2.65:

Day 5; transverse section of a spindle and early anaphase as seen using EM. Numerous microtubules (e.g., arrows) are focussed on kinetochores (e.g., white arrowheads) with sister chromatids (e.g., stars) attached to microtubules from opposite poles. Most organelles such as mitochondria (e.g., M) are excluded from the spindle zone, but present within it are ribosomes, vesicles (e.g., circled) and rough ER (e.g., black arrowhead). The axis of the spindle runs from top left to bottom right of the image. x 15,000.

Figure 2.66:

Day 5; transverse section of a very young cell-plate forming between two telophase nuclei as seen using EM.

(a) Detail; showing remnant spindle microtubules (e.g., arrows) near the re-forming nuclear envelopes (e.g. star). Also present are phragmoplast microtubules (e.g., arrowheads), which lie either side of the vesicles (e.g., asterisk) that will form the cell plate. x 17,000.

(b) View; illustrating the exclusion of larger organelles such as amyloplasts (e.g., A) and mitochondria (e.g., M) from the spindle/phragmoplast area. The developing cell-plate (asterisk) and re-forming telophase nuclei (N) can also be seen. x 10,000.

Figure 2.67:

Serial section, approximately 2µm thick and stained using toluidine blue, of the dividing cell illustrated in Figure 2.66 as seen using A-DIC optics. The forming cell-plate (arrow) lies along the axis of a phragmosome (arrowheads), which, as with recent divisions (e.g., stars), lies parallel to the wound (which would have been across the top of the page). x 400.
Figures 2.68 - 2.70:

Cytokinesis as seen using EM.

Figure 2.68:

Day 5; longitudinal section of a nearly-complete cell plate.

(a) View; the wound would have been up and down the left-hand side of the page and so the two walls visible are end walls. The cell plate has fused with the lower wall (black arrow) whilst at the upper wall the microtubules of the phragmoplast (arrowheads) are still present and fusion (white arrow) is just taking place. A gradient of cell-plate maturation is visible away from the phragmoplast: the nearer to the lower wall the less vesicular and the more wall-like the plate becomes. x 3,000.

(b) Higher magnification of a portion of (a) showing ER (arrowhead) penetrating the cell plate at a site (arrow) which presumably becomes a plasmodesma. x 50,000.

Figure 2.69:

Day 5; transverse section of a periclinal wall that has been inserted in response to wounding. ER (arrowheads) lies either side of a plasmodesma (arrow) and appears to penetrate through it. x 30,000.

Figure 2.70:

Day 5; transverse section showing telophase nuclei (N), either side of a cell-plate (arrow), asymmetrically-positioned within a daughter cell. The wound was positioned to the top left, recent divisions (stars) are visible as is the mother-cell wall (R). x 4,000.
CHAPTER THREE

PITH CELL WALL ARCHITECTURE BEFORE WOUNDING

It is well-accepted that a significant amount of meristematic activity and a rapid change in the basic morphology of the meristem occur in response to injury. Wounding cells of an organ do not respond at high rates by undergoing quick, marker-driven cell divisions. Unlike meristem cells and developing cells, wound-induced changes in the walls of the wound cavity are also induced. These changes in the walls can be important to the wound healing process and can also cause the death of cells. Thus, the wounding of the cut meristem results in a substantial change in the normal development of auxin. Auxin, synthesized in the wounded tissue, is transported in a longitudinal stream of polar auxin vectors to a region of cell division. The auxin then induces a series of longitudinal changes in the walls of cells, resulting in a transverse orientation of the walls. These events are described in detail by (1961). It emerged in the course of the study that in all meristems, the cells responding to a wound stimulus a substantial change in the detailed examination of the cytoplasmic effects of wounding cells. This study has led to two basic observations: (1) the polarly shifted pattern of wall deposition, and (2) the significant changes in the pattern of wall deposition. It is clear that these changes are at least partly due to the polarly shifted pattern of wall deposition, which is in concert with the nature of wall deposition.
3.1 INTRODUCTION

It is well-established that a resumption of meristematic activity and a 90° change in the main direction of tissue expansion occurs in stem pith cells of *D. stramonium* in response to tissue wounding (e.g., Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1983; present study). Chapter 2 of this thesis focussed on the protoplasmic reorganisation that takes place in responding cells before the first round of wound-induced cell division. The substantial concomitant increase in the thickness of the walls of the same cells was also noted. This would seem to be important to the wound reaction because, as first expounded by Frey-Wyssling (1959) and Roelofsen (1959), significant cell expansion tends to occur at right angles to the general direction of cellulose microfibril deposition. More recently it has been demonstrated, in the alga *Nitraria*, that the direction of deposition of the inner (25%) layer of microfibrils has a dominant influence upon the geometry of expansion (Richmond et al., 1980). Thus, the expansion of the typical higher-plant cell maturing in a longitudinal file of cells within an organ, such as a pith cell in a *D. stramonium* stem, is mainly parallel to the file and has cellulose reinforcement normal to it (e.g., Green, 1980). Generalising, any major changes in the direction of expansion, such as the formation of a lateral organ, will require a switch in the direction of cellulose deposition from transverse to longitudinal, in at least some parts of the developing tissues (e.g., Green, 1983; Green and Poethig, 1982; Hardham, 1982).

It emerged in the course of the present work that *D. stramonium* pith cells responding to a wound provide a relatively simple system for the detailed examination of the way in which higher plant cells can alter their pattern of wall deposition, and of how the alterations relate to the polarity shift of predictable nature. In order to document the nature of
the wall deposition after wounding, it was first necessary to determine the
initial status of the pith cell walls. This is detailed in the current
chapter and the wall changes that occur in response to wounding are
described in the following chapter. The succeeding paragraphs provide a
background to the approaches that are described in both chapters.

The birefringence of cellulose microfibrils means that changes in
cellulose deposition can conveniently be studied in polarised light (e.g.,
Preston, 1974). A relevant example of this type of analysis concerns the
shift in growth axis in epidermal cells of a detached leaf of *Graptopetalum
paraguayense*, where a lateral organ forms from a structure with a previous
apico-basal polarity (Green, 1984; Green and Brooks, 1978; Green and Lang,
1981). Micro-dissection techniques were used to remove a sheet of outer
periclinal walls, which was then examined using polarised light to
determine the overall cellulosic order at both the level of the individual
cell and on a more global basis. Related changes in microtubular arrays in
the epidermal (Hardham *et al.*, 1980) and internal tissue (Selker and Green,
1984) were studied using EM.

The studies of Green and co-workers are the only ones to examine in
detail extensive cellulosic changes during an induced alteration in
polarity in higher plants. There have been no reports of the elucidations
of global changes in cellulose status in internal tissue of higher plants
undergoing a 'natural' switch in polarity (i.e., one not induced by factors
such as externally-applied growth hormones, as carried out by Veen, 1970a,
b and Ridge, 1973). On the other hand, a considerable amount of work has
been carried out on the normal development and status of the walls of
parenchyma or pith cells in several species (for reviews see e.g., Preston,
1974; Roland and Vian, 1979; Wardrop, 1962). A serious drawback of many of
these studies is that, by their use of macerated tissue (e.g., Böhmer,
1958; Scott et al., 1956; Setterfield and Bayley, 1958; Wardrop, 1955, 1956), the spatial organisation of the tissue is destroyed in the course of the examination. This disadvantage can be partially obviated by the use of sectioned embedded material (e.g., Ridge, 1973; Wardrop et al., 1979), although problems arise in obtaining large areas of wall completely within the section.

Interpretation of the results of these studies has resulted in some controversy (e.g., compare Preston, 1982 and Wardrop et al., 1979 with Neville and Levy, 1984 and Roland and Vian, 1979) and this may arise, at least in part, from the difficulty of extrapolating from minute samples, as seen by EM, to the level of the whole cell or tissue. It is therefore apparent that, if possible, a combination of several approaches should be used when attempting to elucidate the wall architecture of even the most simple cells.

In the current study the initial state of the pith cells and the early stages of reorganisation were examined by a combination of light and EM. The pith of D. stramonium is fairly uniformly composed of cells that are large enough to permit two types of microsurgical examination. In one procedure thick hand sections were cut and mounted so that designated wall faces of cells from a known position with respect to the wound surface could be isolated for detailed characterisation by polarisation (for retardation) and interference (for optical thickness) optics, the unwanted portions of the preparation having been removed by carving. In the second procedure, cells or cell files were isolated by extraction of fixed tissue with EGTA. Individual cells were cut with razor-blades and micro-knives so that all wall faces could be laid flat on a microscope slide for measurements of their retardation and OPD. The second procedure gave complete portraits of individual cells; the first setting the information
into the context of the whole tissue by preserving the global positional relationships. Both techniques pinpointed areas in which further study by EM was required. The information obtained by the methods as a whole showed the initial construction of the cell walls to be considerably more complex than was originally envisaged (Chapter 3, Results), and also that the walls undergo changes during the polarity shift that were unforeseen (Chapter 4).

In this thesis, as is in other studies of plant cell walls (e.g., Lang et al., 1982), the orientation of microfibrils seen in electron microscopic sections is assumed to be the same as the orientation of the cellulose microfibrils.
3.2 MATERIALS AND METHODS

3.2.1 Preparation of radial and transverse walls

The following technique was developed to examine apposed radial walls (i.e., the composite wall common to two adjacent cells) with polarising and interference optics. Fresh radial longitudinal sections were made by hand using microknives, which were manufactured by a technique modified from Green and Lang (1981). The specimen was immersed in water and gripped in a 'universal stage' (Green, 1984) mounted on a Wild APO M5 dissecting microscope for ease of cutting. Sections were transferred into 2% 'Teepol' and then rinsed twice in distilled water to remove the bulk of the cytoplasm. After air-drying onto microscope slides, the sections were examined under the dissecting microscope and in polarised light. Sections with radial walls lying flat against the slide were photographed at low magnification before further trimming. The tissue surrounding the relevant walls was then removed by hand under the dissecting microscope using a combination of razor-blade and micro-knife. This left each wall in its original location and orientation relative to the wound edge (see Chapter 4) and at the same time rendered the walls suitable for detailed optical study by removing flare produced by less well-oriented surrounding walls. Apposed end (transverse) walls - i.e., those common to the top and bottom of vertically adjacent cells - were prepared in a similar manner, except that transverse sections of the stem were used.

3.2.2 Preparation of 'opened-out cells' from macerated tissue

Pieces of tissue were cut, fixed and rinsed as for EM (Section 2.2.5) except that paraformaldehyde was sometimes omitted from the fixative. The tissue was then incubated at 37°C for a minimum of 2h, but usually overnight, in 50mM EGTA (Sigma Chemical Co., St. Louis, Mo, 63178, U.S.A.) in buffer (25mM potassium phosphate) or distilled water - the latter proved
to be more effective in softening the sample. The tissue was squashed under a 'Teflon'-coated coverslip in 10% (v/v) polyethylene glycol 400 (PEG 400; BDH Chemicals Ltd., Poole, England) in H₂O. The solvent prevented the cells from drying out and the coating prevented them from sticking to the coverslip when it was removed. Upon squashing, cells tended to remain in longitudinal files, presumably reflecting more tenacious links at transverse walls. However, many individual cells were also released. Under the dissecting microscope, such cells were picked out of the squash suspension on the tip of a micro-knife and transferred to a drop of water on a fresh slide. Cells were then cut open and laid flat (Fig. 3.1) by hand using a combination of razor-blade and micro-knife, together with intermittent drying to stick the samples to the glass in a reversible manner, thus aiding the delicate carving procedure. The transverse walls were removed first. Although they are themselves flat, any attached portions of the side wall had to be removed or cut radially to enable the specimen to be completely flattened. The remaining side (longitudinal) wall was cut lengthwise. This created two pieces of side wall which could be unfolded to give a view of the whole wall. In some cases it was possible to cut through just one thickness of side-wall, thus allowing the entire longitudinal faces of the cell to be unfolded as a single piece. Preparations derived from isolated cells in this way consisted of single walls, as distinct from the apposed composite walls obtained by carving hand sections.

3.2.3 Polarised light microscopy

Specimens were examined and photographed (on Kodak Plus-X film) using a Leitz Orthoplan polarising microscope fitted with a λ (first-order red) plate or 1/20 wavelength compensator. Retardation (τ) of individual
specimens was calculated by taking a series of micrographs at differing compensator settings and then determining the angle of maximum specimen darkness. From the relationship \( \Gamma = \Gamma_{\text{max}} \sin 2 \theta \), where \( \Gamma_{\text{max}} \) is 27 if 540nm is taken as the wavelength of white light, the retardation is obtained in nanometres. Specimens were usually mounted in water under coverslips to reduce light-scattering whilst still allowing easy mountant removal for subsequent interference microscopy. PEG 400 which, owing to its high refractive index, enhances the images in polarised light, was sometimes used, but its removal for dry examination in the interference microscope was difficult. Any strain birefringence inherent in the glass was negated by taking a 'zero reading' of the surround for each specimen.

3.2.4 Interference microscopy

The optical thickness of individual specimens was determined using interference optics on a Peraval Interphako microscope (Carl Zeiss, Jena) set up for either uniform- or fringe-field interference (see Beyer [1974] for the details of this microscope), both of which utilise a sheared (double) image (see e.g., Figs. 3.7b, d). In the latter technique a pattern of parallel stripes (fringes) is laid across the field spaced at intervals representing one wavelength of the incident light. The specimen deflects these fringes by a distance equal to its OPD and this deflection can be accurately measured (as fractions of a wavelength) on an enlarged image of a negative (Kodak Tri-X film was usually used). The optical thickness \( (d) \) of the specimen is then determined using the formula \( \text{OPD} = d (n_{\text{object}} - n_{\text{medium}}) \). To obtain the refractive index \( (n_{\text{object}}) \) of the specimen the OPD is measured using air \( (n = 1.00) \) and water \( (n = 1.33) \) as mounting media and then, after obtaining the value of \( d \) from
For example, for the wall shown in Figure 3.2d the OPD in air is 356nm and in water (not illustrated) 116nm. Thus, \( d = \frac{356 - 116}{1.33 - 1.00} = 727 \text{nm} \).

Therefore, \( n_{\text{object}} = \frac{356 + 1}{727} = 1.49 \).

This value is the mean of the refractive indices involved in birefrigence. The \( n_{\text{object}} \) of several walls was determined and the average value (1.49) used in all calculations. The direction and number of fringe displacements was determined in white light, with measurements made in monochromatic green light using a 552nm interference filter. Specimens were usually left unmounted, open to the air, to maximise fringe displacement, which is proportional to \( n_{\text{object}} - n_{\text{medium}} \) and to mass/area of the specimen. Uniform field optics was used to visualise local variations in OPD, which were apparent as a difference either in colour in white light or in contrast when using monochromatic light.

### 3.2.5 Electron microscopy

In addition to examination of conventionally-embedded and -stained tissue, processed as in Section 2.2.5, other techniques were utilised.

In order to examine microfibril orientations at cell corners in more detail, tissue was treated with 1% (v/v) pectinase (P-5146, Sigma Chemical Co., St. Louis, Mo, 63178, U.S.A.) in 0.01M sodium citrate at pH 4 for 8h at RT. After a brief rinse in citrate the tissue was fixed overnight (12 to 18h) in 4% (v/v) glutaraldehyde in citrate prior to dehydration and embedding in Spurr's resin as described in Section 2.2.5. Most sections were stained with 5% (w/v) potassium permanganate for 30 mins (following
Deshpande, 1976a) and rinsed with either distilled water or 0.025% (w/v) citric acid. Remaining sections were conventionally stained (Section 2.2.5). The citrate rinse (as recommended by Lawn, 1960 and Sutton, 1968) reduced the contamination that was introduced by permanganate staining, but this measure was not generally used here, as it resulted in a loss of contrast (as also noted by Bray and Wagenaar, 1978).

To stain pectins within the tissue, samples were processed in a manner similar to that described by Albersheim et al. (1960). After overnight fixation in 4% (w/v) paraformaldehyde the specimens were brought into 60% ethanol prior to 1h in fresh hydroxylamine (made up from equal volumes of 14% (w/v) NaOH in 60% ethanol and 14% (w/v) hydroxylammonium chloride (BDH Chemicals Australia Pty. Ltd., Port Fairy, Victoria 3284, Australia) in 60% ethanol) after which time the mixture was acidified with an equal volume of 1:2 concentrated HCl:95% ethanol. This was removed after 5 min and replaced with 10% (w/v) ferric chloride (May and Baker Ltd, Dagenham, England) in 60%-ethanol containing 0.1M HCl. After 1h the tissue was rinsed in 60% ethanol and dehydrated through an ethanol series into Spurr’s resin. Sections were examined without further staining.

To obtain replicas of opened-out side walls, they were agitated in water using a micro-knife in order to remove the cytoplasm. After transfer to Formvar-coated 50 mesh grids, walls were coated with approximately 2.5nm of platinum/carbon at an angle of 45° followed by a backing coat of approximately 25nm of carbon at an angle of 90° in a Balzer’s freeze-etch apparatus (coating kindly performed by Mr. E. Hines, R.S.B.S., A.N.U.). Following dissolution of the grids and walls in chromic acid, the replicas were rinsed in distilled water prior to picking-up on Formvar-coated slot grids and examination in the electron microscope.
In order to section opened-out side walls 'end-on', i.e., transversely with respect to their original orientation in the stem, they were manoeuvred in a drop of water, using a micro-knife, onto thin bricks of 2% (w/v) agar. These bricks were made by cutting slices from a mound of solid agar and trimming to an oblong shape. The wall was positioned so that it lay in the same orientation as the agar-brick, i.e., with the original long axis of the cell parallel to the short side of the brick. Once the water had evaporated, molten agar was dropped from a loop over the brick to seal the wall in place. After it had solidified, the agar was again trimmed to a block shape, fixed in 2% (v/v) glutaraldehyde and processed for EM as per Section 2.2.5. Upon embedding, the brick was placed with its long side against the edge of the block-mould so that it was in the correct orientation for direct sectioning (Fig. 3.15). After polymerisation, all faces of the brick were smoothed-off to reduce light scattering so that the wall could be located in polarised light (Fig. 3.2). Sections were cut and stained conventionally or with the same solutions, but for 30min each, or using permanganate (as above).
3.3 RESULTS

3.3.1 Longitudinal walls

**Overall transverse order**

Observations in polarised light show that radial longitudinal walls of *D. stramonium* stem pith cells have transverse cellulose reinforcement, as illustrated by the hand-carved wall in Figure 3.2a.

Overall transverse orientation of cellulose was confirmed by EM where congruent transverse microtubules and microfibrils are seen in transverse sections (Fig. 3.3). Transverse order is apparently uniform (within the limits of detection) as retardation ($\Gamma$, measured in polarised light) and optical thickness ($d$, measured by fringe interference optics) vary in parallel when walls of different thicknesses are compared. This relationship is shown in Figure 3.4 where the data points lie about the line representing $-\Gamma$ varying in direct proportion to $d$.

**Pit fields**

Uniform and fringe field images (Figs. 3.2c, d) show that transverse order is also fairly even across individual wall faces, except at the site of pit fields. These are large and lens-shaped, with their long axes parallel to the overall direction of cellulose reinforcement. As revealed by interference microscopy, the pit margins show increased fringe deflection relative to the wall between the pits, indicative of a local increase in OPD (Fig. 3.2d). In fact, circumferential reinforcement occurs at the pit margin, manifested by the appearance of 'Maltese Crosses' centred on the pits when they are placed with their long axis at 90° to the polariser or analyser in polarised light (Fig. 3.2b) and also by their behaviour when a first-order red plate is placed in the light path. A slight rotation of the long axis away from those of the crossed-polars results in a loss of the cross image. This relates to the elongated shape.
of the pits - the more circular an object is, the further it has to be rotated before the cross is lost (Preston, 1974).

In order for this circumferential reinforcement to occur at the margin of the pits, the constituent cellulose microfibrils must behave in one of two ways: (i) as a discrete population running continuously around the margin, or (ii) as a local disturbance of the otherwise transverse reinforcement, flowing around the margins of the pit and crossing-over at its tips in an approximation of circumferential reinforcement. The latter seems more appropriate for polarised light images (e.g., Fig. 3.2a) and replicas do indeed illustrate such a pattern (Fig. 3.6), although some straight microfibrils are also seen to skirt the pit at an angle of approximately 45°.

**Longitudinal ribs**

Longitudinal cell faces dissected individually from hand sections reveal uniform transverse order between the pits, but the more complete samples provided by opened-out cells have an additional feature. Polarisation (Figs. 3.5a and 3.7a) and interference (Figs. 3.5b and 3.7b) views of opened-out cells consistently include bands which run longitudinally along the side walls. They are absent from transverse walls and generally have a retardation only slightly different - sometimes more and other times less - from that of the adjacent wall, but are consistently thicker (Fig. 3.7c). That the thickenings are in the same position as the bands observed in polarised light is demonstrated by the image in uniform field interference (Fig. 3.7b). Rotation of the sample by 90° in the fringe field (Fig. 3.7d) confirms that the thickenings only run longitudinally. The combination of large increases in thickness with only marginally-changed retardation means that these thickenings are qualitatively different from the surrounding side-wall.
The distribution of the longitudinal thickenings in the side walls was checked by measuring wall thickness, excluding the middle lamella, in electron micrographs of cell perimeters (Fig. 3.12). Double thickenings, corresponding to the paired lines seen in interference and polarised light images, coincide with the margins of intercellular spaces. Frequently the thickenings are single, as broad as the combined double ribs, and equivalent in size and location to the whole of the wall face abutting an intercellular space (Fig. 3.7a). This distribution is just detectable in conventional light microscope views of the transverse sections (see Fig. 2.3). As would be expected for a wall facet that is juxtaposed to an intercellular space, pits are excluded from the zone within each paired line, although they may overlap slightly with the wall thickenings themselves.

EM shows that the thickenings result from both internal and external wall specialisations. Externally, a layer of longitudinal fibrils is found at the vertices-(edges) of intercellular spaces (Fig. 3.9). These are positively stained by permanganate, as are the transverse microfibrils in the wall (Fig. 3.12). Internally, conventional staining of the wall along cell edges gives the appearance of longitudinal microfibrillar layers interspersed between the generally transversely-oriented microfibrils (Fig. 3.8). However, positive staining of pectinase-extracted tissue with permanganate (Fig. 3.11) shows that there are no longitudinally-oriented microfibrils in the region of the wall, merely gaps between transversely-oriented microfibrils which spread out at the cell corners. As the external longitudinal fibrils were observed to be omnipresent at intercellular spaces, while at the same time the retardation of these areas is sometimes more negative than the adjacent wall faces, there must also be a greater amount of transverse cellulose at these sites, in addition to the...
spreading of the lamellae. It was observed that pectinase-extracted preparations scarcely stain with the uranyl-lead procedure, suggesting that this conventional method outlines microfibrils by staining matrix materials associated with them. Permanganate staining, by contrast, seems to reveal microfibrils as such (thought to be a physical staining, Deshpande, 1976a).

In this regard, opened-out cells appear to have most, but not all, of the matrix extracted by the EGTA incubation. This emerges because, when sectioned for EM (Fig. 3.14a), they barely stained with conventional uranyl-lead (which did heavily stain remnants of cytoplasm that were sometimes seen attached to the wall), but they did stain with long (30 min) incubations. It may be inferred that a large proportion of the non-cellulosic substances had been removed. These images (Fig. 3.14c) and those of permanganate staining (Fig. 3.14b) show the transverse lamellae interspersed with areas containing only the (remaining) matrix. As with the pectinase-extracted tissue, slight separation of the transverse lamellae appeared to have occurred, even away from intercellular spaces, the sites of which could be judged by the appearance of a putative splitting layer (after Roland, 1978) on the outer side of the wall, i.e., the opposite side to the remnant cytoplasm. These splitting layers were limited to five places along the opened-out wall (marked with asterisks in Fig. 3.14a), a number of sites which would be expected for intercellular spaces (see Section 2.3.3). They were also observed in pectinase-extracted tissue at intercellular spaces (only) (Fig. 3.11).

That pectins are present throughout the wall in the region of intercellular spaces (and indeed all other regions) was demonstrated by ferric ion staining of pectin hydroxyamic acids, formed by substituting hydroxylamine for the methyl groups of pectin (Fig. 3.10). This reaction has been shown to be specific for pectins (Rougier, 1971 and references therein).
The picture that emerges is that the marked increase in OPD at the bands is caused by a combination of the external longitudinal microfibrils and the extra transverse internal reinforcement. Their relative amounts determine the direction and magnitude of the change in retardation at the bands compared with the adjacent wall faces.

3.3.2 Transverse walls

In strictly transverse walls, no overall order is seen in polarised light, either in walls dissected from hand sections (Fig. 3.17) or in walls derived from cut-open isolated cells (Fig. 3.7a). Although neutral as a whole, local ordering of groups of microfibrils is indicated by the patches of light and dark seen in compensated images. The relative disorder, as compared with the side walls, is also apparent in the pits of transverse end walls. They are small, roughly circular and, when viewed between crossed polars, exhibit a Maltese Cross with only very weak retardation, and then not at all points of the compass. Grazing views of transverse walls in the electron microscope (Fig. 3.16) confirm the lack of overall order and the general absence of circumferential reinforcement around the pits. Ordered groups of microfibrils with congruently-aligned microtubules are apparent, but they pass in random directions across the transverse walls, skirting or traversing pits.

Oblique end walls differ significantly from strictly transverse walls. This was initially detected because some of the end walls from hand sections differed in appearance from those described above (Fig. 3.18a). Comparisons were made by opening out selected cells in isolated cell files possessing internal transverse walls and terminal oblique walls. One such oblique wall is shown in polarised light (Fig. 3.20a) and uniform- (Fig. 3.20b) and fringe- (Fig. 3.20c) field interference, still attached to portions of the side wall of the same cell. The pits are slightly larger
than in transverse walls, lie in lines with detectable birefringence between and parallel to the lines (Figs. 3.18a and 3.20a) and exhibit more obvious Maltese Crosses when viewed between crossed polars (Fig. 3.18b). These features were also observed in oblique apposed pairs of end walls dissected from the middle of files of cells (Fig. 3.19). It is apparent that oblique end walls possess a proportion of the features of side walls, including at least some overall transverse order.
3.4 DISCUSSION

In order to investigate wound-induced changes in cell wall development and structure, it was necessary to adopt methods that could provide as much information as possible about cells at a particular location in the tissue, i.e., where polarity shifts occur prior to the cell divisions that ultimately produce the wound cambium. The technique of trimming away unwanted material from thick hand-sections provided the identification and position of the selected walls, and subsequent polarising and interference microscopy provided information as to their molecular make-up. Examination of opened out, isolated cells then permitted integration of these data to the level of the whole cell. Both techniques pinpointed the specific localities where clarification by EM was necessary. While still incomplete, the observations do permit the changes that follow wounding to be interpreted (Chapter 4).

*D. stramonium* pith cells exhibit a considerable degree of spatial control over their wall deposition. They are not simply transversely-reinforced cylinders, although the longitudinal cell-faces are indeed fairly uniformly transversely-reinforced, exhibiting disturbance only locally at the site of pits. Their 'hoops' of reinforcement are augmented by additional microfibrillar material at the cell edges and at the margins of intercellular spaces. Gaps appear between groups of microfibrils at these locations: EM shows them to be occupied by pectic and probably other matrix materials. Judging by their staining characteristics as seen using EM, these matrix substances appear to be extracted by the EGTA used to isolate the cells, hence their contribution to the OPD of the longitudinal ribs (measured on EGTA-isolated cells) is likely to be small. The separation of the transversely-oriented microfibrils into distinct lamellae that occurred with both the EGTA- and pectinase-extracted tissue in the
current study, was also observed in parenchymatous cell walls of *Cucurbita*
petioles that had been extracted by pectinase and other substances
(Deshpande, 1976b). Furthermore, in the same study, it was noted that the
number and thickness of the lamellae was greater at cell corners than at
the intervening wall faces. A similar form of local wall thickening has
been observed in other species and cell types where the gaps between the
microfibril layers were also thought to consist mainly of pectins (Cork,
1980).

The cell edges differ further from the faces of the cell in that they
carry externally-positioned microfibrillar ribs which consist of a layer of
exclusively longitudinally-oriented microfibrils. The external thickenings
do not spread far laterally onto the cell faces, as demonstrated by their
sharp delineation in polarised light images. Uniform field interference
images reflect the more diffuse nature of the extra internal transverse
thickenings. Thus optical thickness increases and decreases relatively
smoothly, whilst retardation changes abruptly at the site of longitudinal
external ribs. The thickenings terminate at the apical and basal ends of
the longitudinal walls and do not encroach upon the end walls, although in
some instances they may circumscribe them. Transverse end walls have
random cellulose orientation with only local order and small pits and can
be seen to differ from the side walls in both polarised and interference
images. Oblique end walls, on the other hand, are only readily
distinguished from the side walls by interference microscopy. They
resemble side walls in polarised light because transverse cellulose hoops
extend from the side walls across the oblique ends.

These observations are in general agreement with, and extend, earlier
studies on parenchymatous cell walls; work which was mainly aimed at
elucidating the mechanisms of cell wall deposition and growth, especially
in the context of the multi-net growth hypothesis of Roelofsen and Houwink (1953) (reviewed by Wardrop, 1962). In general, these earlier studies accord with the conclusion reached by Setterfield and Bayley (1958) about the structure of parenchymatous cell walls of *Avena* coleoptiles: 'Walls of these cells consist firstly of regions containing the primary pit fields and composed of microfibrils oriented predominantly transversely. Between the pit field regions and running the length of the cells are ribs composed of longitudinally-oriented microfibrils'. These ribs were initially thought to be internal secondary thickenings (Wardrop, 1955, 1956), but were later realised to be on the outer surface and so part of the primary wall (Bohmer, 1958; Setterfield and Bayley, 1958, 1961; Wardrop and Cronshaw, 1958). However, Setterfield and Bayley (1958) also observed longitudinal ribs through the whole thickness of the wall with, apparently, no internal transverse deposition (integral ribs). While deposition of longitudinal microfibrils may occur in young cells (see below), there are no other reports of longitudinal ribs occupying the complete thickness of the wall in mature tissue. The technique used by Setterfield and Bayley (1958) involved shadowing extracted, methacrylate sections in which microfibrils could possibly become displaced, and so the appearance of integral ribs may be an artefact of preparation. Indeed, in a later study of the same system (*Avena* coleoptile), Wardrop *et al.* (1979) only observed a layer of longitudinal microfibrils at the external side of corner thickenings. In none of the above studies were complete cells opened out to facilitate examination of all the walls of individual cells. When isolated walls were used they were invariably from macerated tissue and so their origins could not be determined. Furthermore, although the external longitudinal 'thickenings' are described, in no case was interference optics used to demonstrate that they are actually thicker. Pairing of the
ribs was either not seen or not commented upon (see, for example, Fig. 2, Plate 1 of Wardrop, 1955).

The present results identify the location of the ribs as longitudinal cell junctions where there are intercellular spaces. Paired ribs lie along the edges of intercellular spaces. Certain implications and possible interpretations follow from this. The most important of these concerns the mode of formation of the ribs. Their longitudinal microfibrils, as the most external layer, must have been deposited early in the development of the cell. This deposition could have occurred when longitudinal cell files were forming from thin-walled precursor cells, either as part of a general longitudinal layer in the side walls or in restricted hoops in association with longitudinal PPBs, which would mark the site of future cell junctions. However, the latter explanation does not seem to be able to account for all junctions even though thickenings appeared to be omnipresent at mature corners. On the other hand, the longitudinal microfibrils may have been laid down as discrete entities during otherwise transverse reinforcement. In this regard, in a freeze-fracture study of parenchyma of maize mesocotyls, Mueller and Brown (1982a) commented that, 'Ribs of microfibrils with longitudinal orientations often are observed on plasma membranes on either side of pit fields [and therefore at cell corners, Scott et al., 1956; present study]. Curving configurations of plasma membrane microfibrils...demonstrate that microfibrils of a longitudinal rib are continuous with those of a transverse orientation between pit fields'.

Roelofsen (1959) has postulated that walls under longitudinal stress (or rather strain - Preston, 1952) deposit longitudinal microfibrils. Thus, corners in young cells may have different strain characteristics from the adjacent cell faces, causing microfibrils to be deposited with a
different orientation. However, while this could account for the presence of ribs in very young cells, as observed by Mühlethaler (1950), such ribs would seem unlikely to remain prominent during subsequent cell expansion. Furthermore, succeeding deposition of transversely-oriented microfibrils (with additional matrix along the cell edges, as in *D. stramonium*) would be internal to the longitudinal microfibrils and so the ribs could not be supplemented by direct deposition from the plasma membrane. Thus, any microfibrillar contribution to the ribs must come from material deposited earlier. In this regard, Wardrop and Cronshaw (1958) considered that during extension growth, microfibrils initially present on the cell faces could be pulled into the longitudinal corner thickenings. Roelofsen (1958, 1959) has proposed a not-too-dissimilar mechanism whereby cell material is squeezed away from the faces of cell contact to the areas of non-contact (intercellular spaces). This is analogous to the way that liquid in a soap bubble film under pressure tends to migrate away from the contact area. As pointed out by Wardrop (1962), such explanations account for the lack of thickenings in hair cells.

These explanations could account for the persistence (Böhmer, 1958; Mühlethaler, 1950; Scott et al., 1956; Wardrop, 1955) of external longitudinal ribs during extension growth, but, owing to their requirement for considerable passive reorientation, not for their initial deposition in young cells. Thus, a synthesis of the above hypotheses that would account for both the initiation and the development of the external longitudinal ribs seems appropriate, *viz.*: in young meristematic cells longitudinal microfibrils are laid down at cell corners and these, upon continuous transverse deposition around the side walls of the cell, become external and are maintained during extension growth by movement and passive reorientation of microfibrils from the adjacent cell wall faces. Indeed,
Roelofsen (1959) himself stated, 'This hypothesis is quite compatible with the possibility that the accumulation of cellulose in the ribs...is partly effected by other processes'.

At present, the ribs' mode of deposition can only be conjectural. However, their longitudinal orientation could favour the local expansion that must occur when intercellular spaces form, whether by splitting, combined with a shape change, or by local growth, or a combination of the two. A group of longitudinal fibrils on the outer face of the wall could serve as a focus for the origin of the intercellular space and for its shaping. Roland (1978) has identified a putative 'splitting layer' in the primary wall which is resistant to chemical extraction and lines intercellular spaces before and after opening. Although his study mentions 'corner reinforcements' he shows no pictures of longitudinal fibres, presenting only transverse sections of the walls in such areas. There is evidence from EM of both the pectinase- and EGTA-extracted tissue that a comparable layer also occurs in *D. stramonium* pith. The 'splitting layer' may initiate separation at the junctions of appressed cells, while the longitudinal microfibrils would allow further expansion of the space.

The longitudinal ribs interrupt what is undoubtedly the major feature of *D. stramonium* pith cells in the wound response-zone - the predominant transverse reinforcement of their longitudinal walls. This alignment 'spills over' onto oblique end walls, where the pits are intermediate in appearance between those of side and transverse walls and are often elongated parallel to the cellulose. Only in strictly transverse walls is the tendency towards hoop reinforcement lost, randomly-arranged bundles of microfibrils instead giving rise to only very local order and approximately circular pit areas.
Mueller and Brown (1982a, b) proposed, on the basis of freeze-fracture images of microfibril imprints in the plasma membrane, that the main factor responsible for the disposition of microfibrils around pit areas was unidirectional membrane flow aided by microtubule guidance. The authors noted that, as can be seen in the polarised light and replica images of *D. stramonium* obtained in the current study, 'Although microfibril orientations are deflected around pit fields, the main axis of microfibril orientation is maintained across the surface of the cell'. Mueller and Brown (1982a) also observed that the most recently deposited microfibrils formed asymmetric patterns around pit fields with no cross-over at putative upstream or downstream extremities. This is in sharp contrast to the image in Figure 3.6 where the pattern is not asymmetrical and a marked crossing-over of microfibrils occurs at the tapered margins of the pit. At first sight this would appear to be incompatible with the postulated unidirectional membrane flow. However, the replica of the wall shows several layers of deposition while the plasma membrane images obtained by freeze-fracture represent a single layer. The disparate images may be reconciled if unidirectional membrane flow does occur, but in two directions (both transverse with respect to the long axis of the cell but 180° apart) at different times. In this regard, some microfibrils can be seen to be deflected at 45° rather than 'flowing' around the pit (Fig. 3.6), but only one way from each end of the pit. It is conceivable that deposition associated with flow from one direction goes 'above' the pit and that from the other goes 'below', rather than 'above' and 'below' from both directions. Mueller and Brown (1982a, b) observed a well-defined border between the plasma membrane against transverse walls (complex microfibril patterns) and the plasma membrane against longitudinal walls (transverse order). This was also observed during the current study. They also found
some microfibril impressions that were continuous from longitudinal to transverse cell faces, akin to the spillover from transverse to oblique walls observed in polarised light in *D. stramonium*.

In the present system, in all categories of wall, it is important to note that the orientation of cortical microtubules mirrors the orientation of the microfibrils seen near the plasma membrane. Further attention is given to this point in Chapter 4, which describes dramatic shifts in microtubule orientation correlated with deposition of microfibrils in orientations that are novel, these effects being an early component of the overall wound response.
In all compensated polarised light micrographs presented, one axis on the key shows the approximate direction of overall cellulose alignment in wall faces that are darker than the background (D); the other axis shows the alignment in wall faces that are lighter (L). In some specimens remnants of wall not lying flat against the slide are present, these scatter light and thus appear flared. All non-isotropic specimens were examined with their long axis at 45° to the crossed-polars (brightest position), except for samples visualised using crossed-polars only, in which case the long axis of the specimen was placed parallel to the polariser or analyser.

In the interference micrographs, part or all of a second image of the specimen may be present; the generation of a sheared image is a feature of the microscope used (see Section 3.2.4). In all fringe field interference micrographs the direction of fringe displacement in the specimen, which is opposite in the two sheared images, is indicated by the white truncated-arrow. In all the samples presented in this chapter the deflection is less than one wavelength.

Otherwise, the labels on the figures follow the same conventions as used in Chapter 2.

**Figure 3.1:**

Diagrammatic representation of the procedure used to open-out an isolated cell (see also text, Section 3.2.2).

The end walls are removed (a), laid down with the inner surface up (b) and flattened with the aid of radial cuts (c). The remaining side wall (d) is cut lengthwise either though both radial walls to give two pieces of side wall (e) or through one wall only, enabling the wall to be unfolded as a single piece (f). The positions of cuts are indicated by the short-curved arrows.
Figures 3.2 - 3.5:

Figure 3.2:

Hand-carved longitudinal wall from the pith of *D. stramonium*.

(a) As seen compensated in polarised light, showing the reasonably uniform transverse reinforcement across the face of the wall except at the margins of pits (e.g., arrow), which are slightly brighter than the surrounding wall as they are not fully compensated. The pattern of the image at these sites is suggestive of extra reinforcement located around the edges of the pits and crossing-over (e.g., arrowhead) at their ends (see also Fig. 3.6).

(b) When viewed under cross-polars, Maltese Crosses are seen (e.g., arrows) centred on the pits, this is indicative of a form of circumferential reinforcement at these sites.

(c) In uniform field interference, the wall, apart from a small fold in the top right-hand corner, has similar contrast across its face owing to a fairly constant optical thickness. The margins of the pits (e.g., arrow), however, are of different contrast reflecting a local difference (increase) in optical thickness.

(d) In fringe field, the constancy of optical thickness across the face is shown by the even displacement of the fringes. Similarly, the deflection of the fringe is locally greater in the region of the pits (e.g., arrow). Overall, these images show that the longitudinal walls from unwounded stems are transversely reinforced with cellulose in an even manner and possess localised thickening at the margin of pits. (a) -(d) x 525.

Figure 3.3:

Electron micrograph of a conventionally-stained transverse section of a longitudinal wall showing transverse cellulosic reinforcement and transverse cortical microtubules (arrows) in the thin layer of cytoplasm. x 40,000.

Figure 3.4:

Plot of retardation (as measured in polarised light) versus optical thickness (determined using interference optics) for individual hand-carved side-walls from the pith of *D. stramonium*. The points lie about the short-dashed line which represents retardation varying in direct proportion to optical thickness, thus confirming the uniformity of transverse reinforcement in walls of different thickness. The circled point represents the wall seen in Fig. 3.2.

Figure 3.5:

The majority of the longitudinal walls of a pith cell isolated by EGTA extraction and cut open.

(a) In polarised light, paired longitudinal lines of different contrast (and therefore retardation) are apparent. Pits are excluded from the darker central zone de-limited by each pair of lines.

(b) In uniform field interference (the sample has been rotated through 90° compared with (a)), the paired lines are of different contrast from the rest of the wall, thus identifying them as being of different (greater) optical thickness. (a) x 165; (b) x 115.
Figures 3.6 and 3.7:

Figure 3.6:

Replica (shadowed from the top right-hand side) of the inner face of a pit in a longitudinal wall face from a cut-open cell isolated by EGTA extraction. The majority of the cellulose microfibrils flow around the pit crossing-over at its tapered margins. However, some straight microfibrils are deflected at an angle of 45° (e.g., arrowheads) above (from the left) and below (from the right) the pit. The extra deposition is superimposed over the general transverse alignment (see also Fig. 3.2), which is visible in places (e.g., arrow). x 10,000.

Figure 3.7:

Pith cell isolated by EGTA extraction and cut open. The side walls are virtually complete, but a portion of one end wall has been lost during the dissection procedure. Both end walls retain small portions of attached side wall.

(a) In polarised light, showing the overall transverse reinforcement of the side walls and the presence of bands of slightly differing retardation (e.g., arrows) running the length of the side walls, but not encroaching upon the end walls (E), which have no overall cellulosic order (similar contrast to the background). The bands are broad, occupying the same sites (at intercellular spaces) as the paired lines seen in Figure 3.5.

(b) In uniform field interference, showing that the bands (e.g., arrows) are of different optical thickness from the adjacent, pitted areas of wall. End walls show no such bands.

(c) In fringe field interference, showing the increased fringe deflection and therefore greater optical thickness at the longitudinal bands (examples of positions marked by black arrows) compared with the adjacent wall. End walls, by contrast, show no such pattern and possess less optical thickness than the side walls, as demonstrated by the difference in fringe deflection at the boundary of the end wall and attached side wall (black arrowhead).

(d) As (c) except that the specimen has been rotated through 90° relative to the fringes. The straightness of individual fringes along the length of the side walls illustrates that the thickened bands are solely longitudinal. (a) and (c) x 160; (b) and (d) x 125.
Figures 3.8 - 3.13:

Electron microscopy of internal and external wall thickenings at intercellular spaces.

Figure 3.8:
Conventionally-stained transverse section at the edge of an intercellular space (I) adjacent to the wall face shown in Figure 3.3. Illustrated are groups of transversely-oriented microfibrils (e.g., white arrows) interspersed with matrix materials, which stain in a particulate manner (e.g., black arrow). The particles are not transverse sections of longitudinal microfibrils (see also Figs. 3.9, 3.11 and 3.13). x 40,000.

Figure 3.9:
Conventionally-stained longitudinal section showing longitudinally-oriented fibrils (black arrow) lining an intercellular space (I). Adjacent to these is an amorphous area (asterisk) presumably composed of matrix materials and some passively re-oriented microfibrils (see Fig. 3.13). Further away from the intercellular space transverse microfibrils are visible (e.g., white arrow). x 30,000.

Figure 3.10:
Transverse section at an intercellular space (I) processed according to a procedure modified from Albersheim et al. (1960) in order to stain pectins, showing that pectins occur throughout the thickness of the wall at these regions. Staining is more dense, not only, as would be expected, in the middle lamella region (asterisk), but also in the most recently deposited (inner) portion of the wall (arrowheads). x 40,000.

Figure 3.11:
Permanganate-stained transverse section of pectinase-extracted tissue at the site of an intercellular space (I) showing the local separation of groups of transverse microfibrils. No longitudinally-oriented microfibrils occur in the gaps (e.g., asterisks) between these lamellae (confirmed by stereo electron microscopy - not shown). Also present is a densely-staining, pectinase resistant layer lining the space (arrowhead). x 15,000.

Figure 3.12:
Plot of longitudinal wall thickness, excluding the densely-staining middle lamella, around a pith cell as measured from electron micrographs of a transverse section. Gaps correspond to pits; asterisks mark the position of intercellular spaces. It can be seen that wall thickness increases at such sites, often in a double-peak fashion, which corresponds with the paired lines seen in polarised light and interference images (e.g., Fig. 3.5). Figures 3.3 and 3.8 are taken from this cell.

Figure 3.13:
Permanganate-stained oblique section of pectinase-extracted tissue at the site of an intercellular space (I), showing the discrete population of longitudinally-oriented microfibrils lining the void (black arrow). Owing to the angle of sectioning these appear as lines perpendicular to the transverse microfibrils on the inner side of the wall (white arrow). In between, microfibrils of mixed orientations occur (asterisk) presumably representing those that have been passively reoriented. x 25,000.
Figures 3.14 - 3.16:

Figure 3.14:

The longitudinal walls from an opened-out cell isolated by EGTA extraction that has been embedded in agar, processed for EM, and sectioned transversely.

(a) Low magnification view of the whole length of the wall in a permanganate-stained section. Five putative splitting-layers (after Roland, 1978) were observed, the positions of which are marked by the asterisks, on the opposite side of the wall to the occasional remnant patch of cytoplasm (not shown). The splitting layers are presumably at the site of intercellular spaces (see also Figure 3.11).

(b) Higher magnification image of a portion of (a) at the site of a splitting layer (arrowhead). The lamellae of transversely-oriented microfibrils are apparent (e.g., white arrows), interspersed with layers of mainly matrix-material (black arrow). Extraction with EGTA is thus not as effective as with pectinase (cf. Fig. 3.11). The retiform structure visible below the splitting layer is the result of staining of the agar plinth.

(c) A serial section of the same area of wall (splitting layer marked by the arrowhead), but conventionally stained for a total of an hour. Transversely-oriented microfibrils are again apparent (e.g., white arrow) as are some matrix materials (e.g., black arrow). This image confirms that some matrix remains after EGTA extraction, as the conventional staining procedure, even for extended periods, barely stains pectinase-extracted tissue (see Fig. 3.11). However, a significant proportion of the matrix-materials would have been removed by the EGTA treatment as serial sections stained for shorter periods exhibited very low contrast in the electron microscope (not shown). (a) x 525; (b) and (c) x 40,000.

Figure 3.15:

Opened-out longitudinal wall from a cell isolated by EGTA extraction as seen in polarised light, after embedding for EM. Subsequent sectioning would have taken place in an orientation perpendicular to the page and across its width in order to produce transverse sections - as in Fig. 3.14a (from a different wall). The longitudinal thickenings are still visible (e.g., arrow). x 55.

Figure 3.16:

Conventionally-stained transverse section showing a grazing view of a transverse end-wall. Groups of straight, parallel microfibrils with congruent microtubules (arrows), are seen traversing the field in many directions and skirting around a roughly circular pit (P). Underlying endoplasmic reticulum (ER) with associated polyribosomes and a putative actin filament bundle (arrowhead) are also present. x 35,000.
Figures 3.17 - 3.20:

End walls.

Figure 3.17:

Three transverse end walls isolated from a hand section, as seen in polarised light, showing their overall random nature with only local order. The pits are extremely small and roughly circular (e.g., white arrows). Patches (e.g., black arrow) of side wall are also present, mainly lying in end-on view. x 340.

Figure 3.18:

An oblique end wall isolated from a hand section.

(a) As seen in compensated polarised light. Compared with transverse end walls (Figure 3.17) the pits (e.g., white arrow) are larger and appear to be in lines. Small remnants of side walls are also present (e.g., black arrow).

(b) When viewed between crossed-polars each pit (e.g., arrow) possesses a Maltese Cross, indicating a degree of circumferential reinforcement at the pit margins. By contrast, transverse end walls exhibit only very weakly retarding, inconsistent Maltese Crosses (not shown). (a) and (b) x 340.

Figure 3.19:

Polarised light image of an oblique (O) and a transverse (E) end wall dissected from a cell in the middle of a file that had been isolated by EGTA extraction. The appearance of the transverse wall matches that of the walls in Figure 3.17, with small pits (e.g., arrow) and little order. The oblique wall, on the other hand, has pits which gradually change in appearance from those characteristics of side walls (e.g., black arrow) to those similar (e.g., white arrow) to the pits in Figures 3.18a and 3.20a. x 285.

Figure 3.20:

The end cell from a file of EGTA isolated cells with an oblique terminal-wall. During dissection, the transverse end-wall was removed and the oblique wall (O in (a)) left in situ attached to the side walls, which were cut through one thickness only and unfolded. The points marked with an asterisk in (a) would have originally been adjacent to each other in the intact cell as would the points marked with a star.

(a) In polarised light, it can be seen that the oblique walls resemble those in Figures 3.18a and 3.19 with some cellulosic order and pits intermediate in size between those of transverse end-walls and side walls. At least some of the cellulosic order spills over from the adjacent side walls (arrowhead).

(b) In uniform field interference, in contrast to the image seen in polarised light, the oblique wall is well-defined (arrowheads). The thickenings that are apparent in other opened-out cells (Figures 3.5 and 3.7), are less marked in this case (large truncated arrows).

(c) In fringe field interference, the edge of the oblique wall is marked by a localised increase in optical thickness (e.g., arrow). This again differs from the image in polarised light where there is only a gradual change of contrast in the same region ((a)). (a) x 400; (b) and (c) x 300.
CHAPTER FOUR

PITH CELL WALL ARCHITECTURE AFTER WOUNDING

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4.1 INTRODUCTION

The cytoplasmic basis of the change in polarity and the resumption of meristematic activity during the wound response of *D. stramonium* pith cells was detailed in Chapter 2. These data demonstrated that a large number of cells is involved and that changes occur in a spatial as well as a temporal gradient. In Chapter 3 methods were described which, capitalising on the relatively large size of the pith cells, provided a description of the construction of the walls in cells prior to wounding. In the present chapter these techniques, which allow convenient polarisation, interference and electron optical analyses of individual cell faces of known position and history, are utilised to examine change in wall architecture during the wound-induced polarity shift.

As in the case of cells in their normal state, the structural details of the responding cells were more complex than was expected. It was presumed that a shift to longitudinal deposition would occur in the radial longitudinal walls and, if 'hoops' of reinforcement were involved, in the transverse end walls also. This shift would favour a change in the direction of expansion from longitudinal to transverse (cf. Green and Poethig, 1982) It was also anticipated that cortical microtubules, which have been shown to mirror cellulose changes during a wound-induced polarity shift in epidermal cells (Hardham *et al.*, 1980), might undergo a comparable shift, moving to lie in the same plane as the periclinal PPB and subsequent new wall (Section 2.3.6).

These expectations were realised, but additional features were superimposed. Firstly, all the walls of the cell, to a greater or lesser extent, were involved in the new pattern of deposition. Secondly, this pattern was not simply longitudinal, but was of polylamellate construction. The nature and mode of development of polylamellate cell
walls is presently a contentious issue. Numerous papers have been written on the subject (e.g., Bentrcia, 1984; Bougiland, 1972; Chafe and Doohan, 1972; Chafe and Wardrop, 1972; Emons, 1982; Emons and Wolters-Arts, 1983; Itoh and Shimaji, 1976; Lang et al., 1972; Lloyd, 1984a; Neville and Levy, 1984; Neville et al., 1976; Pilet and Roland, 1974; Pluymaekers, 1982; Preston, 1982; Reis, 1981; Reis and Roland, 1974; Reis et al., 1982; Roland, 1981; Roland and Mosiniak, 1983; Roland and Vian, 1979; Roland et al., 1975, 1977, 1982, 1983; Sargent, 1978; Sassen et al., 1981; Satiat-Jeunemaître, 1981, 1984; Sawhney and Srivastava, 1975; Vian, 1981; Vian et al., 1982; Wardrop et al., 1979). The unexpected development of a polylamellate wall during the wound response necessitated detailed investigation, particularly to elucidate whether the constituent cellulose microfibrils are deposited in a helicoidal (as per Neville and Levy, 1984; Roland and Vian, 1979) or cross-ply manner (as per Wardrop et al., 1979). Study of the distribution of cortical microtubules was also necessary, as it has been claimed - in some cases rather prematurely (Neville et al., 1976) - that microtubules do not exert a major influence on the control of the direction of cellulose microfibrils in polylamellate walls. Instead, it has been proposed (Roland and Vian, 1979) that this type of wall self-assembles from a liquid crystalline phase (Bougiland, 1972), controlled via an endogenous ('ultradian') rhythm (Roland, 1981; Roland et al., 1983) which can be modified (a) by the plant to vary wall structure and (b) by environmental or hormonal influences (Reis et al., 1982; Roland et al., 1983; Vian et al., 1982). Accordingly, the present work was extended to include examination of the effect of a constant environment upon the polylamellate wall structure. Another aspect of the work described in this chapter is an attempt to elucidate which facets of the wall (and cytoplasmic) wound-response are associated with the change in polarity, as
distinct from the resumption of meristematic activity. This involved comparison between longitudinal wounding (with polarity shift) and transverse wounding (decapitation; with meristematic activity, but no change in polarity).

The terms transverse, longitudinal, radial, periclinal and anticlinal, as used throughout, refer to the geometry of the wounded internode. Thus, the pith cells that were examined had completed their predominantly longitudinal extension and had transverse cellulose alignment in their walls; they respond to wounding by initially depositing longitudinally-aligned cellulose and expanding in a predominantly radial (anticlinal) direction and dividing periclinaly.
4.2 MATERIALS AND METHODS

4.2.1 Specimen preparation following conventional (longitudinal) wounding

Descriptions have been given of the growth and harvesting of plants (Section 2.2.1), wounding (2.2.2), the preparation of radial and transverse walls (3.2.1), the opening of cells from macerated tissue (3.2.2), polarised light microscopy (3.2.3), interference microscopy (3.2.4), and conventional EM (2.2.5). There was no change in the average refractive index \( n = 1.49 \) of the cell walls during the period after wounding and so calculations of OPD remained as for unwounded walls.

Cortical microtubule distribution was determined by making complete montages of the perimeter of responding cells, usually at an instrument magnification of 15,000x. The cells are so large that each one required about 50 micrographs for complete coverage. Microtubules were identified from these micrographs and their position and orientation marked on low magnification diagrams. Even though relatively few cells were scored in such a complete manner, the observations of the pattern of microtubule orientation were repeated many times in other cells from different blocks and different wounds. Similar montages were used for measuring wall thickness around cells.

Stereomicroscopy of ultra-thin sections was accomplished using a side-entry goniometer stage on a Hitachi H-600 electron microscope.

4.2.2 Wounding and responses in plants grown in a constant environment

Seedlings were spiked out and grown either as described previously (Section 2.2.1) or with constant illumination at 25°C in a growth cabinet. Upon wounding, all plants were placed in the same constant light and temperature environment. Harvesting and subsequent processing for conventional EM (Section 2.2.5) were carried out 0, 2 or 4 days after wounding.
4.2.3 Transverse wounding

4.2.3.1 Wounding

Transverse wounding was carried out on plants at the same stage of development as was chosen for longitudinal wounds (Section 2.2.2). Plants were decapitated, using a sterile scalpel-blade, 1cm below the petiole of the leaf immediately below the crotch. Tissue was harvested 0, 1, 2, 3, 4, 5 and 12 days after wounding.

4.2.3.2 Hand sectioning

Longitudinal sections were cut by hand in a similar manner to that for longitudinal wounds (Section 2.2.3.1) except that 4% (w/v) paraformaldehyde in 25mM potassium phosphate buffer was sometimes used as a fixative. Sections were examined fixed or unfixed and stained (with toluidine blue) or unstained using DIC optics.

4.2.3.3 Polarising microscopy

Longitudinal sections of the wound zone were cut and examined in polarised light as in Section 3.2.3. As no large-scale interference study was required (see Section 4.3.6), trimming of the specimens so as to leave individual walls was not usually carried out.
4.3 RESULTS

4.3.1 Architecture of longitudinal cell-walls after wounding

The change in cellulose deposition in the post-wounding period may be introduced by referring first to the condition that is reached after 4 days when cell divisions commence. By then the radial walls have thickened substantially, as shown by the increased fringe deflection in interference images (Fig. 4.9b). The slightly undulating nature of the fringes indicates that the additional wall-material is not deposited as evenly as it was in unwounded walls (cf. Fig. 4.9b with Fig. 3.2d). The walls have also lost their overall transverse reinforcement and have neutral or longitudinal (positive) reinforcement (Fig. 4.9a). Since there was generally little or no expansion by day 4 (Chapter 2), this implies that cellulose deposition is predominantly longitudinal after tissue wounding. This change is further illustrated by the pits. They change in form from a transversely-elongated lens-shape to roughly circular. When rotated in polarised light some maintain a Maltese Cross at all orientations. This, combined with their behaviour when a first order red plate is used in place of the compensator, is indicative of some form of circumferential reinforcement at the pit margin (Preston, 1974). This would be expected to occur if a lens shape composed of longitudinal microfibrils was superimposed over the pre-existing transverse lenticular pit (cf. Fig. 6 of Ridge, 1973). Lack of new cross walls in many parts of these day 4 specimens indicates that deposition has altered before the completion of the first round of cell division.

Transition from the day 0 condition to that at day 4 (and subsequently) was examined on a day-to-day basis. Quantitative results are summarised in Figures 4.1 - 4.5, where retardation (nm) is plotted against optical thickness (µm) for populations of radial walls sampled by carving
on each day. The radial walls were often not as uniform as they were before wounding and so retardation and optical thickness sometimes varied slightly across the face of individual specimens. In these cases, each plotted point represents the average values obtained for a single wall.

Using the line representing varying in direct proportion to d from Figure 3.4 as a reference, it can be seen that by day 1 (Fig. 4.1) little alteration in thickness or retardation has occurred from the unwounded condition. By day 2 (Fig. 4.2) there is a conspicuous drop in retardation, especially in those cells where a change in pit shape is visible (Fig. 4.11).

Day 3 walls (Fig. 4.3) differ even more markedly and fall into two main groups. In the first there has only been a slight drop in retardation and the pits are little changed. The second group consists of cells that have responded by showing a large increase in thickness and developing a birefringence close to neutral. It is therefore apparent both from these analyses and from anatomical observations (Section 2.3.5) that the cell response is fairly heterogeneous. The day 3 specimen shown in Figures 4.13 a-f (which also serve to illustrate how unwanted tissue is removed, leaving the walls of interest in situ) reveals one source of this heterogeneity. It is apparent that cells immediately under the wound (Figs. 4.13c and d) show little change; light (Section 2.3.5) and EM (not illustrated) demonstrate that the cytoplasm of these cells exhibits the early stages of the wound response, but then the cells die. This also appears to be the case with their walls: there is a slight response before the cells die. Such cells comprise the first group of points in Figure 4.3. Internal to this moribund zone is an area of maximal cytoplasmic response, which diminishes as distance from the wound increases (Section 2.3.5). This distribution also seems to hold true for wall thickening, except that
sectioned material (Section 2.3.5), as well as carving data, shows that it occurs to a greater depth within the tissue than does subsequent mitotic activity. The responding cells (Figs. 4.13e and f) yield the second group of points in Figure 4.3. The data for these cells illustrate (i) the predominance of longitudinal deposition in the thickened, responded (but not yet divided) cells; (ii) the concomitant change in sign of birefringence and (iii) the associated change in pit shape.

By day 4 (Fig. 4.4) a third group of points representing walls with reduced thickness appears. These are from cells which have undergone expansion after division. The dramatic thinning that occurs when the mother-cell wall expands (e.g., Figs. 2.44 - 2.48) is even clearer by day 7 (Fig. 4.5) where expanded walls are thin and have an overall birefringence near to zero (Fig. 4.12). The mother-cell walls can easily be recognised; the day 7 results described here referring to them and not to new radial cross-walls, many of which have by then appeared in the tissue.

The above changes in the radial wall were related to the other walls of the cell by examination of opened-out cells from wounded stems. As expected, by day 4 (Fig. 4.17a) counter-deposition has occurred, reducing retardation to around zero. However, this is non-uniform, being more marked in two expanses within the composite opened-out longitudinal walls. The areas of greater change match the appearance of the carved radial (anticlinal) walls. The areas of lesser change equate with periclinal walls. It was shown in Chapter 2 (e.g., Figs. 2.47 and 2.48) that there is a marked difference in the expansion that is undergone by the radial-longitudinal walls and the tangential-periclinal walls during the first few rounds of wound-induced divisions. The increase in thickness caused by additional deposition on radial walls is shown by the greater deflection of the fringes (Fig. 4.17d) compared with unwounded tissue (Fig.
3.7c). Fringe deflection is less than that observed in the 'carved' walls because cells isolated by EGTA maceration are separated from their neighbours at the middle lamella.

Despite the large amount of deposition the thickened bands seen in the pre-wounding cells still remain visible in uniform field interference images (Fig. 4.17b). Just as in the period before wounding, at no stage do the ribs pass from the side walls to transverse end walls. Furthermore, rotation of the sample by 90° in the fringe field (Fig. 4.17c) confirms that the thickenings only run longitudinally.

Examination of opened-out cells from days 1, 2 and 3 show a gradual transition in wall characteristics between those of day 0 and those of day 4. Little change is apparent by day 1 (Figs. 4.14a, b), but by day 2 (Figs. 4.15a-c) a change in pit shape is apparent as is the beginning of a difference in the response across the faces of the cell. By day 3 (Figs. 4.16a-c) a greater change in two portions of the overall side wall is discernable (Fig. 4.16a), as is the concomitant increase in thickness (Fig. 4.16c).

4.3.2 Architecture of transverse cell-walls after wounding

Views of opened-out cells (Figs. 4.14 - 4.17) show that transverse walls also respond in the post-wounding phase. They increase in thickness (Fig. 4.6), but, as shown by views of several day 2 (Fig. 4.18a) and day 4 (Fig. 4.19a) stages, retain their original low birefringence, indicating lack of overall cellulosic order. However, the pits develop Maltese Crosses with fairly strong retardation (Figs. 4.18b and 4.19b), which is maintained at all orientations under crossed polars. Also, many of the samples (e.g. Fig. 4.19b) are divided into quadrants thus giving them an overall cross-shape.
It appears therefore, that post-wounding deposition in transverse walls must be qualitatively different from that occurring in their pre-wounding state. This is confirmed by EM, where it is apparent that there is layering in the end wall material that has been laid down during wounding (Fig. 4.20). Furthermore, some of these layers seem to be continuous with longitudinal post-wounding layers in adjacent side walls of the same cell (Fig. 4.21). If a spill-over of longitudinal 'hoops' from the side walls is the cause of post-wounding deposition in the end walls, the layers must be deposited so as to maintain the low retardation of the specimens. This could occur if radial wall deposition continued over the end wall and then periclinal deposition did the same. This would give: (i) overall cancellation of birefringence in the end walls, (ii) circumferential reinforcement of their pits and, probably, (iii) the weak cross-shape visualised.

4.3.3 Polylamellate wall structure

Changes in cell wall architecture were further examined by conventional EM of longitudinal walls. Prior to wounding, transverse microfibrils appear as lines in transverse sections (see Fig. 3.3), whereas by day 1 (Fig. 4.22) a small amount of longitudinal deposition is apparent as a layer of dots (end views) between the original transverse microfibrils and the plasma membrane. That this deposition is purely longitudinal and is accomplished by an abrupt change in the direction of deposition is shown by longitudinal grazing views of radial walls. In the example shown in Figure 4.23 the associated cortical microtubules are transversely-oriented, presumably as a prelude to the second (transverse) layer of deposition. By day 4 (Fig. 4.24) the wall has become thickened and polylamellate with apparently alternating layers of longitudinally- and tranversely-oriented
bands of cellulose microfibrils. Measurements of the thickness of the original transverse, and post-wounding transverse and longitudinal, layers could be made from micrographs of day 4 radial walls in responding, but not yet divided, cells, in which all layers were readily distinguishable. In all samples the recent (post-wounding) longitudinal deposition was greater than the transverse. When the overall orientation was determined (pre- and post-wounding layers), converted into µm, and then translated into approximate retardations (using the line representing -rad in Fig. 3.4), the values lay close to neutral. This accords with the polarised light data, which indicated progressive neutralisation of the original transverse order (Figs. 4.1 - 4.3).

That the post-wounding deposition consists of alternating transverse and longitudinal layers with abrupt changes between them is shown in images obtained by stereo EM. It has been proposed that polylamellate walls are laid down in a helicoid fashion with gradual changes in the direction of wall deposition in successive layers (e.g., Neville and Levy, 1984; Roland and Vian, 1979) and that such structures appear as switching parabolic arcs when viewed in tilted sections. In the system under study here no such arcs are seen and layer-to-layer changes are accomplished by major switches in the direction of wall microfibril deposition. In all the earlier stages of wall deposition, such as the day 2 radial wall seen in longitudinal section in Figures 4.26a-c, these switches are through an angle of 90°. Most evident are the alternate transverse layers which, upon tilting, simultaneously change from dots to short lines and thus contain microfibrils aligned parallel to each other. However, in a few radial walls, some of the later layers to be deposited may have an intermediate (45°) layer present (Figs. 4.25a-c). Nevertheless, no gradual shift between lamellae is apparent, as also illustrated by grazing longitudinal
sections of radial walls where several layers of post-wounding deposition have taken place (Fig. 4.28). The longitudinal layers (Figs. 4.25a-c) behave in a similar manner to the transverse layers in Figures 4.26a-c) and in stereo views give no hint of gradual helicoidal transitions.

The post-wounding tranversely-oriented layers of microfibrils are similar to the original and presumably consist of microfibrils that pass across the longitudinal faces of the cell. Indeed, these layers could be traced around cell corners, as in the day 4 radial/periclinal junction in Figure 4.27. This was also the case for longitudinal layers, which passed from side to end walls (Fig. 4.29).

By day 4 an average of 9 lamellae had been deposited after wounding. The timetable of deposition has not been followed in detail, but plants subjected to a constant light and temperature environment (Section 4.2.2) also developed polylamellate walls in response to wounding (Fig. 4.30).

After the deposition of a few lamellae of a polylamellate wall, some cells, immediately under the dead cells beneath the cut surface, developed a layer (Fig. 4.31) which EM appeared to show was mainly composed of suberin (cf. Figs. 51a, b of Frey-Wyssling, 1976). This suberised lamella was not uniform around the walls of such cells, being mainly located against the outer periclinal wall. Few radial walls with suberin deposition were observed in the electron microscope and so the days 1 to 4 (Figs. 4.1 - 4.4) radial wall data for little-changed walls presented in Section 4.3.1 represent sub-wound cells where cytoplasmic death has occurred, rather than those with suberised walls. However, in a few of the (day 7) carved radial walls from expanded mother-cells there were patches of wall which, when viewed in polarised light (Fig. 4.10), exhibited overall transverse order, but were considerably thicker than the surrounding wall (Fig. 4.7) and had circular pits. (In Fig. 4.5 such pits
have been categorised as 'slightly reversed' owing to their illustrations of 'Maltese Crosses' with weaker retardation than those shown by pits from walls with neutral or positive retardation.) These islands appeared to be an integral part of the expanded wall and, judging by the presence of cross-walls (Fig. 4.10), often overlapped several of the daughter cells. Such patches presumably arise from the occasional suberised radial wall that has been torn (cf. Steward and Muhlethaler, 1953) by expanding non-suberised neighbours. The tearing would occur because microfibrils encrusted with suberin are inextensible (Muhlethaler, 1961). Although suberin itself is isotropic, the waxes embedded in it are negatively birefringent (Muhlethaler, 1961) and this would give the patches the appearance of overall transverse order. The cellulose initially laid down longitudinally after wounding, but prior to suberisation, would account for the change in pit shape.

4.3.4 Longitudinal ribs

The distribution of longitudinal thickenings during the wound response was checked by measuring wall thicknesses in electron micrographs. Samples were categorised as 'new' or 'original' thicknesses. In Chapter 3 it was reported that, in unwounded cells, ribs of thickening coincide with the position of the intercellular spaces (see Fig. 3.12). Furthermore, double 'peaks' are often observed which correspond to the paired lines seen at the margins of intercellular spaces in polarised light. By day 1 (Fig. 4.7), some deposition has occurred, seemingly all in a longitudinal manner (Fig. 4.22 shows part of the cell for which measurements are given in Fig. 4.7). The original pattern of peaks (thickenings) at the intercellular spaces has been maintained. By day 4 (Fig. 4.8), a massive post-wounding increase in wall thickness is apparent (Fig. 4.24 is taken from this
cell). However, the peaks remain in the same position as they occupied before wounding, thus confirming that the thickenings observed in the EGTA-opened cells have been maintained. There is a preference for deposition in the radial rather than the periclinal walls (quite apparent in the inner periclinal, but less clear in the outer), again conforming with observations made with the polarising microscope.

Figures 4.7 and 4.8 provide information on two individual cells, but the patterns of deposition they reveal were observed in several other cells of similar stages (data not shown).

4.3.5 Cortical microtubule distribution

Prior to wounding, the cortical microtubules against longitudinal walls lie in a transverse orientation (see Chapter 3). The change to polylamellate wall organisation must involve microtubules changing orientation if they participate in spatial control of wall deposition. What actually happens is complex and may be summarised as follows. (i) In none of the responding cells scored were microtubules exclusively running in the same direction around the circumference of the cell. (ii) Mirroring of microtubule patterns in opposite walls of the same cell was usually observed, combined with marked differences between anticlinal and periclinal walls; i.e., if the radial walls had mainly longitudinally-oriented microtubules, the periclinal walls had the majority of their microtubules running in a transverse direction (Figs. 4.32 and 4.33). The opposite combination of orientation was also seen (Fig. 4.34). (iii) Although not so distinct at the early stages of the response (Fig. 4.32), in every case observed at the later stages, individual cell faces were virtually uniform in respect of microtubule orientation (Figs. 4.33 and 4.34).
4.3.6 Transverse wound response

In a trial experiment, examination of several decapitated day 12 plants revealed that a wound response had occurred. All plants possessed a moribund layer of variable, but sometimes quite considerable, depth. Under this tier was a zone where several divisions, mainly parallel to the wound, and some expansion, had taken place. Both division and expansion were much less than would have occurred with longitudinal wounds of the same age (Chapter 2). In addition, there was no evidence of the tissue re­differentiation seen in longitudinal wounds. The above observations led to an investigation of the early stages of the pith-cell response to transverse wounding. For convenience the responses in both the walls and the cytoplasm are given here.

The unwounded and day 0 condition of the pith cells was the same as that described previously (Section 2.3.3). By day 1 amyloplast clustering had taken place in some of the cells (Fig. 4.35), but was more prevalent by day 2 when most of the cells underlying the wound had organelle-enclustered nuclei. The speed of this response is in marked contrast with that in longitudinal wounds, where enclustering was virtually omnipresent after one day and, in some cases, after only 8 hours (Section 2.3.5). Most apparent in transverse wounds was a lack of traumatotaxis: organelle-enclustered nuclei being found adjacent to all sides of the cells. This absence of nuclear migration to the wound wall was unequivocally demonstrated by day 3 wounds, where the first manifestation of systrophic formation, namely the appearance of fine cytoplasmic strands radiating from the enclustered nucleus to the other walls of the cell, was observed at nuclei against all walls of the cell (Fig. 4.36). These subsequently formed cytoplasmic stars (Fig. 4.37) identical in appearance to those of longitudinal wounds. The first round of divisions occurred by day 4 in many of the cells. The divisions were at 90° to the stem axis. Mitosis occurred in a phragmosome
parallel to the transverse wound (Figs. 4.38a, b), forming new transverse walls (Fig. 4.39), some of which (Fig. 4.39 and 4.40) lined up with those in adjacent cells. Subsequent rounds of division followed the same pattern of events with cytoplasmic strands focussing on daughter nuclei prior to systrophic formation (Fig. 4.40) followed by division, usually parallel to the wound.

In marked contrast to the events after longitudinal wounding, there is no extra cellulose deposition in the pith cells in response to transverse wounding. The lack of change in pit shape in the day 3 specimen in Figure 4.41 demonstrates that no longitudinal deposition had taken place. That no significant transverse deposition had occurred, was shown by the retention of pre-wounding retardation values. This is further illustrated by the day 5 specimen seen in Figure 4.42, where undivided cells slightly away from the wound match the appearance of moribund cells at the cut surface (cf. Fig. 4.13a). Preliminary observations using interference optics revealed no obvious change in the distance of fringe deflection in walls at day 3 compared with those at day 0.

After several rounds of division (Fig. 4.43), some expansion of the mother-cell walls had taken place, thus reducing their retardation to around zero. It can also be seen that the majority of the divisions were parallel with the wound and that cells which are immediately under the dividing zone have changed little in appearance.
4.4 DISCUSSION

In the following, unless otherwise stated, the wound response under discussion is that following conventional (longitudinal) wounding.

Previous studies of wound responses in dicotyledonous stems have concentrated on factors involved in tissue determination (Benayoun et al., 1975; Snow, 1942; Warren Wilson, 1978; Warren Wilson and Warren Wilson, 1961a, b, 1963, 1983; Warren Wilson et al., 1983) and the issue of whether cell division is a pre-requisite for (e.g.) phloem differentiation around a wound (Behnke and Schultz, 1980 - cf. with Hardham and McCully [1982a, b] for pea roots). Changes that take place within the cells before divisions commence have not been examined in detail. It is apparent from the results presented in this chapter that important changes in cell wall deposition occur in *D. stramonium* pith cells that are responding to a nearby wound, before divisions have started. These changes anticipate the 90° shift in the plane of division and major axis of extension.

Prior to wounding, the pith cells have transverse cellulose reinforcement around the side walls of the cell, with external, longitudinal, reinforced thickenings at the cell edges. Transverse end walls are random in their overall microfibril orientation. Oblique end walls exhibit some transverse order as a 'spillover' from the side walls.

The transverse-wound response results in a reactivation of meristematic activity, but does not require a change in cell polarity and no alteration of cellulose deposition occurs. In such wounds, the prelude to the first round of division contains all the cytoplasmic events observed after longitudinal wounding, except for traumatomaxis. This feature, therefore, is not associated with the organisation of the division plane, but is, at least, a manifestation of the change in polarity (see Section 2.4). A link between traumatomaxis and the other early indicator of a
change in polarity, an alteration of wall deposition, is shown by the occurrence of both phenomena to a greater depth than subsequent mitoses in longitudinal wounds. Indeed, post-wounding wall deposition was not observed in cells in which nuclear migration had not taken place. It would be of interest to use cytochalasin treatments in attempts to discern whether traumatotaxis is in fact a pre-requisite, rather than merely a correlate, of the re-polarisation events of wall deposition and cytokinesis.

During the wound response, polylamellate deposition of alternating layers of microfibrils occurs around the side walls of the cell, with each layer approximately normal to the preceding one and with an overall bias towards the longitudinal orientation. Combined with the original transverse order, this leads to a composite structure which, although it may possess neutral birefringence, is far from randomly structured. Similar layering in transverse end walls seemingly results from the formation of 'hoops' of microfibrils passing either periclinal or anticlinally over the side (longitudinal) walls of the cell and tangentially or radially (respectively) over the end walls (see Fig. 4.44). This change in the end walls from disordered to ordered deposition is of interest as, prior to wounding, they would have expanded little; but after wound-induced divisions occur, they expand greatly in the radial direction.

However, it is not immediately apparent how the wound-induced polylamellation of all the walls in the cell is related to subsequent expansion. Expansion is predominantly radial and cell division is predominantly periclinal. It cannot simply be that expansion is normal to the orientation of the cellulose reinforcement, as was first generalised by Frey-Wyssling (1959) and Roelofsen (1959) and later reviewed by Roelofsen.
(1965) and Preston (1974). In a number of cases it was found that the most recent layer of reinforcement in *D. stramonium* cells which had reached the stage of mitosis, or possessed a newly-inserted cell plate, was periclinal, i.e., passing longitudinally over the radial walls and tangentially over the transverse walls. It could therefore be the case that these cells behave in the manner of *Nitella* internodal cells, shown by Richmond *et al.* (1980) to expand in axes that are influenced by the microfibril orientation of the innermost (most recent) part of the wall. However, even if the innermost layer does not have an overriding influence, there are several other examples in which polylamellate walls expand (e.g., Chafe and Wardrop, 1972; Roland and Vian, 1979; Wardrop *et al.*, 1979) and it may be that the expansion geometry of cells with polylamellate walls is related to the overall balance of the successive layers (Lang *et al.*, 1982). Alternatively, such walls may be isotropic with regard to constraints upon expansion and, as the stress is primarily transverse in an elongate cylinder like a *D. stramonium* pith cell, the main direction of extension would be transverse (Green, 1963). Furthermore, it seems likely in the case of the responding *D. stramonium* pith cells that the restraints on expansion imposed by the non-responding tissue surrounding the wound area allows only outward radial growth.

Wardrop *et al.* (1979) have extended the multi-net growth (now termed 'passive reorientation' by Green, see Preston, 1982) hypothesis of Roelofsen and Houwink (1953) to account for the expansion of polylamellate (collenchyma) walls. These authors considered that, in longitudinally-oriented layers, the microfibrils would move relative to each other, that in transverse layers reorientation of microfibrils would occur, and thus, with expansion, the layers would thin. In the current study, the thinning of the mother-cell walls that occurs upon expansion has been documented quantitatively.
It has been advocated that passive reorientation plays no part in wall expansion and that polylamellate walls similar to the one formed in the present system, are helicoidal, arising from gradual changes in the angle of pitch between successive layers of microfibrils (Neville and Levy, 1984; Neville et al., 1976; Reis et al., 1982; Roland, 1981; Roland and Mosiniak, 1983; Roland and Vian, 1979; Roland et al., 1977, 1982, 1983; Vian et al., 1982). This interpretation is inconsistent with the apparent simple 90° switch from longitudinal to transverse and vice-versa seen in the present work. It is to be noted that the responding D. stramonium pith cells lay down their alternating wall layers before any cell expansion occurs, hence distortions introduced by stretching (c.f. Sargent, 1978) can be discounted.

The response of the cells includes the following key features, in addition to those discussed above. (i) In all responding cells studied, walls opposite to each other (i.e., radial opposite radial, inner periclinal opposite outer periclinal) have similar current microtubule orientations and histories of wall-layering. (ii) Microtubules against radial walls with recently-deposited longitudinal microfibrils are almost exclusively longitudinal, whilst those against the periclinal walls in the same cell at the same time have a high proportion which are transversely-oriented (e.g., Fig. 4.33). The converse is also true (Fig. 4.34). Microtubules, with congruent wall microfibrils, are thus present at both sets of opposite wall faces. (iii) Even though the amount of deposition varies around the side walls of the cell, being greatest in the radial walls, slightly less in the outer periclinal and much reduced in the inner periclinal, the number of layers is the same or very similar and they appear to be continuous from one wall to the next. This is akin to the situation in epidermal cells where the transverse lamellae of the thickened
outer walls thin as they continue into the adjacent radial walls (Chafe and Wardrop, 1972). (iv) The general pattern of expansion of the wound matches, to a large degree, the extent of deposition in the side walls and, indeed, the end walls - which also exhibit considerable deposition after wounding. (v) The switches in orientation are relatively abrupt, although the existence of small numbers of microfibrils with an intermediate orientation between the orthogonal layers - as was observed in collenchyma by Chafe and Wardrop (1970) - in the later layers to be deposited in the radial and end walls cannot be excluded. However, only 90° switches were observed in the periclinal walls.

The phenomenology described above is depicted in Figure 4.44. The initial, pre-wounding, state includes 'hoops' of microtubules (the hoops may consist of short interdigitating microtubules passing transversely around the cell). Microtubules of transverse end walls are randomly oriented in the plane of the wall. Wounding initiates formation of a hoop of microtubules normal to the initial transverse girdle. The new, longitudinal, hoop is continuous around two of the side walls and the end walls. Sometimes it is anticlinal and at other times it is periclinal, this switch being fairly abrupt, as inferred from the sudden changes in microfibril orientation seen in the post-wounding transverse walls. (Paradoxically, were the changes from wall face to wall face not abrupt and the longitudinal hoop of microtubules did in fact gradually rotate about its long axis a geometrically helicoidal wall, arising not through self-assembly, but through microtubule mediation, would form on the end walls.) Side wall faces revert to the initial, pre-wounding state, with transverse microtubules when they are not occupied by the longitudinal hoop. These transverse microtubules and congruent microfibrils correlate with the transverse layering of the polylamellate wall, each transverse layer being
completed as the longitudinal hoop vacates periclinal or radial walls, successively freeing them for reversion to the transverse orientation. This possession of a 'memory' position for transverse cortical microtubules has been implicated in many other systems; not least during reinstatement of microtubule arrays after mitosis (Green and Poethig, 1982), but also in cells recovering from colchicine treatment (Taiz et al., 1981). The rotation of the longitudinal hoop of microtubules could be related to the movement of higher plant protoplasts relative to the cell wall that has been suggested by Mueller and Brown (1982a).

Superimposed on the overall spatial controls there is a quantitative variation in the extent of wall deposition: radial walls > outer walls > inner periclinal. This has not been analysed here in detail in respect of microtubule frequency, but there are many precedents for variation in their numbers and orientation (as well as in wall deposition) around the individual faces of cells (e.g., Busby and Gunning, 1983).

Abrupt or gradual changes in microtubule orientation have been observed both in normally-developing cells and in cells submitted to experimental manipulation. The best-studied example of endogenous switching is in the unicellular alga Oocystis (see Quader and Robinson, 1981), where, as in D. stramonium pith, the successive changes seem to be abrupt. This is also the case with another alga, Boergesenia, in which the number of layers formed in the polylamellate wall in constant light is double that produced in constant darkness, although microtubule mediation is not thought to be responsible (Mizuta and Wada, 1982). In D. stramonium pith, both the pre- (not illustrated) and post-wounding walls from plants grown in a constant environment appeared identical to those grown in a changing environment. This constancy of response means that any endogenous rhythm must be relatively stable (cf. Roland et al., 1983). Various
hormonal treatments can introduce changes in microtubule orientation (reviewed by Gunning and Hardham, 1982, see also Mita and Shibaoka, 1984a, b, c) and alterations in the time spent depositing the successive layers of polylamellate walls, with correlated shifts in the axis of cell expansion. It seems likely that the large-scale effects seen in the wound response of *D. stramonium* pith are mediated by changes in hormonal régimes (Warren Wilson, 1978; Warren Wilson and Warren Wilson, 1983). On the cellular level, ethylene treatment introduces shifts in microtubular orientation (Steen and Chadwick, 1981), probably by a gradual process that may involve rotation of the favoured axis rather than an abrupt change (Lloyd et al., 1984). It is notable that, in ethylene treatments, the shift in microtubule orientation is not exclusive: 10% of microtubules remaining in the non-predominant transverse orientation in treated tissues (Lang et al., 1982). The intracellular distribution of these unshifted microtubules was not studied and it may be that, as in *D. stramonium* pith cells, they represent a population lying against walls where deposition of a 'hoop' of reinforcement was not occurring at the time when the tissue was fixed and which has reverted to a 'ground state', favoured perhaps by a 'memory' in the form of a thermodynamically-stable state (Green, 1984). In this regard, in a recent paper originating from Roland's laboratory (Satiat-Jeunemaître, 1984) it was observed that parenchymatous cell walls of *Zea mays* coleoptiles developed, after treatment with ethylene, a polylamellate wall over the previous 'unilayered texture' (i.e., transversely-aligned microfibrils). The techniques used prevented investigation of cell organelles and the result was discussed in terms of the modification of the ultradian and endogenous rhythms that are held to be partially responsible (e.g., Roland et al., 1983) for the control of wall morphogenesis. However, as mentioned above, treatment with ethylene
has been reported (e.g., Lang et al., 1982) to cause cortical microtubules to assume a longitudinal orientation. Therefore, the wall pattern observed in treated tissue by Satiat-Jeunemaître (1984), could also be accounted for by a rotating longitudinal hoop of cortical microtubules (combined with possession of a transverse 'memory position') as is postulated to occur in pith cells of *D. stramonium* during their wound-response.

Wound-induced shifts in microtubule orientation have also been examined in detached leaves of *Graptopetalum*, where the epidermis (Hardham et al., 1980) and subepidermal cells (Selker and Green, 1984) become involved in organogenesis. In *D. stramonium* the shifts clearly precede cell division, but in *Graptopetalum* they seem to follow division and to be geometrically-related to the plane of division. Microtubules with mixed orientations occur at individual cell faces during transitional periods (Hardham et al., 1980; Selker and Green, 1984), reflecting the maintenance of microtubule and cellulose microfibril congruency (see Lloyd [1984a] for a recent review on such relationships). The results of the present study also point to a very close link between the disposition of cortical microtubules and the pattern of the forming polylamellate wall.

It is unclear whether the organisation of wall deposition is related to the subsequent division plane. A longitudinal hoop of microtubules encompassing the radial and end walls would be in the same orientation as the subsequent PPB. However, in one day 4 cell the polylamellations in the outer periclinal wall were at 45° to the long axis, but still at 90° to each other, and yet the cell had recently divided transversely. (This was the only cell observed whose initial post-wounding lamellae were not longitudinally- and transversely-oriented.)

In addition to the intracellular symmetry observed in the post-wounding deposition in *D. stramonium* pith there may well be some form of
intercellular synchrony. Adjacent appressed radial walls are often strikingly symmetrical, with the number and thickness of longitudinal and transverse layers mirroring each other in the two cells to a large degree (Figs. 4.22 and 4.24). This mirroring suggests that either direct intercellular communication is occurring or that the adjacent cells are reacting in the same manner to a common external stimulus. The possible appearance of 45° layers in the radial walls as they thicken could therefore stem from either a breakdown in communication with increased thickness (in this regard no 45° layers were observed in the thinner, periclinal walls) or to a modification of the effect of the external influence (stress?) by the extra deposition. As already described (Section 2.3.5), intercellular co-ordination is also manifest in another phenomenon - the continuity of the line of new cell walls across the responding tissue. The 4-way junctions at new cell walls and the symmetry in respect of the numbers of wall layers both speak of control networks that transcend the boundaries of individual cells and extend in three dimensions throughout the wound zone.
The conventions for polarised light and interference images are as described in Chapter 3, except that in some of the fringe field interference specimens presented here fringe deflection is more than one, but less than two, wavelengths. In these cases, the direction of fringe deflection within the sample is marked by two, rather than one, white truncated-arrows. Otherwise, all abbreviations and conventions for the figures presented are as used in previous chapters.

Figures 4.1 - 4.4:

Plots of retardation (as measured in polarised light) versus optical thickness (determined using interference optics) for individual hand-carved radial walls 1, 2, 3 and 4 days after wounding (for day 7, see Figure 3.5). The short-dashed line is derived from Figure 3.4 and represents retardation varying in direct proportion to optical thickness. The long-dashed line represents zero retardation; above it walls have overall longitudinal cellulosic reinforcement, below the line they have overall transverse reinforcement. "Pits normal" refers to pits having the appearance in polarised light identical to those in walls from unwounded tissue (e.g., see Fig. 3.2a), i.e., having a lenticular shape elongated normal to the long axis of the wall and, when viewed under crossed polars, a Maltese Cross that disappears upon rotation of the sample away from the axis of the polariser or analyser. "Pits reversed" is used to describe pits which have, owing to considerable longitudinal deposition, lost their lens-shape and have become roughly circular (e.g., Fig. 4.9a) and exhibit a Maltese Cross at all points of the compass when viewed under crossed-polars. Pits intermediate in appearance between those regarded as typical of the unwounded (pits normal) and those of the fully-responded (pits reversed) state are classified as "pits slightly reversed".

Figure 4.1:

By day 1, little alteration in optical thickness or retardation from the unwounded condition is apparent (see Fig. 3.4), although the pits of most samples have slightly changed in appearance.

Figure 4.2:

A conspicuous drop in retardation has occurred in day 2 samples, many of which exhibit a considerable change in pit shape, such as those in the walls shown in Figure 4.11 (circled points).

Figure 4.3:

Day 3 walls fall into two main groups. The first shows only a slight drop in retardation and the pits have changed little in appearance. Figure 4.13c (point enclosed by a square) is within this cohort. The second group of walls have thickened considerably and have birefringence close to neutral, such as the wall shown in Figure 4.13 (circled point).

Figure 4.4:

In addition to the two contrasting types of wall seen at day 3 (Figure 4.3), a third group of points representing walls with reduced thickness is present. These are from cells that have undergone expansion after division (see Figure 4.5). The circled point represents the wall shown in Figure 4.9.
Figures 4.5 - 4.8:

**Figure 4.5:**

As Figures 4.1 - 4.4, except 7 days after wounding. Most walls have expanded, thinned and exhibit retardation very close to neutral, such as the sample shown in Figure 4.12 (circled point). However, some thinned walls possess areas of considerable optical thickness and negative retardation (see Fig. 4.10, which is represented by the points enclosed by a square and a triangle).

**Figure 4.6:**

Plot of optical thickness of end walls (as measured using interference optics) at days 1-4 after wounding. Optical thickness increases markedly after wounding in a statistically significant manner. Student t-tests of the data revealed that, at the 0.95 level, day 4 walls had a significantly greater optical thickness than days 2 and 3 samples and that these, in turn, had a significantly greater optical thickness than the days 0 and 1 specimens. There was no significant difference between either the day 0 and day 1 walls or the day 2 and day 3 walls.

**Figure 4.7:**

Plot of longitudinal wall thickness around a responding pith-cell at day 1 as measured from electron micrographs of a transverse section. Measurements of both pre- and post-wounding deposition could be made because the boundary between them could be easily identified (e.g., see Fig. 4.22, which is taken from this cell). As in Figure 3.12, the densely-staining middle lamella was not included. Asterisks mark the position of intercellular spaces; OP the outer periclinal wall; IP the inner periclinal wall; and R the radial walls. It can be seen that deposition has occurred all around the cell and that the original pattern of single or double peaks of thickness at the site of intercellular spaces (see Fig. 3.12) has been maintained.

**Figure 4.8:**

Plot as per Fig. 4.7, except of data from a day 4 cell. The gap represents the position of a pit. It can be seen that considerable deposition has occurred, but in an uneven manner around the cell, being least prominent in the inner periclinal wall. Despite the post-wounding deposition, the peaks remain at the site of intercellular spaces, thus confirming that the thickenings observed in opened-out cells (see Fig. 4.17) are maintained. Figure 4.24 is taken from this cell and its microtubule layout is shown in Figure 4.33.
Figures 4.9 - 4.13:

Hand-carved radial-walls after wounding.

Figure 4.9:

Hand-carved radial-wall from a day 4 wound.

(a) In polarised light (cf. Fig. 3.2a), the wall face mainly exhibits longitudinal cellulosic reinforcement. The pits (e.g., arrow), even in this compensated image, possess Maltese Crosses and have lost their pre-wound lens shape.

(b) In fringe field interference, fringe deflection (approximately 1.8 wavelengths) is considerably greater than before wounding (see Fig. 3.2d). The slightly uneven displacement of the fringes across the wall shows that the post-wounding deposition is not completely uniform. (a) and (b) x 525.

Figure 4.10:

Hand-carved radial-wall from a day 7 wound as seen in polarised light. This is an expanded mother-cell wall and the majority of it has thinned (determined by interference optics, see Fig. 4.5) and has neutral birefringence. However, one portion has remained thickened and exhibits negative retardation. This islet contains a pit (white arrow), which, although circular, only exhibits a Maltese Cross with weak retardation when viewed under crossed polars (not illustrated). The patch of wall, which traverses several daughter cells as judged by the presence of cross-walls (black arrow), is presumed to be a portion of suberised wall that has been torn during expansion (see text, Section 4.3.3). x 525.

Figure 4.11:

Hand-carved radial-walls from four adjacent cells from a day 2 wound as seen in polarised light. The pits (e.g., white arrow) have changed appearance from the unwounded state much as in Figure 4.9a. However, cellulose alignment is still transverse overall. Bright portions of attached end and periclinal walls still remain (e.g., black arrow) and these prevented accurate interference measurements from being made in two of the cells. Data for the other two are given in Figure 4.2. x 525.

Figure 4.12:

Hand-carved radial-wall from a day 7 wound as seen in polarised light. An expanded mother-cell wall as in Figure 4.10, except that no suberised patches are present. x 525.

Figure 4.13:

Hand-carved radial-longitudinal-section from a day 3 wound. (a), (b) and (c) as seen in polarised light: axes are the same in all images (see (b)).

(a) Prior to further trimming. It can be seen that there is a dramatic difference in overall cellulose orientation between cells immediately under the wound (W) and those several files away from it. The bright lines are periclinal- and end-walls oriented perpendicular to the page.
(b) After trimming, unwanted tissue has been removed, leaving the walls of interest in situ.

(c) The wall from immediately under the wound, its appearance is very similar to that of walls from unwounded tissue (e.g., see Fig. 3.2a) with transversely elongated pits (e.g., arrow) and negative retardation (transverse order).

(d) The same cell as in (c), but as seen in fringe field interference. Fringe displacement is similar to that of unwounded tissue (cf. Fig. 3.2d) and much less than in (f). This wall is shown by the point enclosed by a square in Figure 4.3.

(e) Responded walls several files under the wound as seen in polarised light (axes as in (b)). Cellulose orientation is longitudinal overall and the pits (e.g., arrow) exhibit Maltese Crosses and have become roughly circular.

(f) The walls in (e), but as seen in fringe field interference. Fringe deflection (just over one wavelength) is greater than in (d), reflecting the considerable wall deposition that has occurred after wounding. The upper cell is represented by the circled point in Figure 4.3. (a) x 60; (b) x 135; (c) - (f) x 325.
Figures 4.14 - 4.16: Pith cells isolated by EGTA extraction and cut open; days 1 to 3.

Figure 4.14:

A nearly-complete day 1 cell.

(a) In polarised light, the image is very similar to that of unwounded cells (see Fig. 3.7a). The side wall possesses lenticular pits (e.g., white arrow) transverse order and longitudinal bands (e.g., black arrows) whilst the end walls (e.g., E) lack both order and bands. The side wall thickenings are not particularly prominent, but this was sometimes observed in unwounded samples and arises because there is only a small difference between the amount of extra interval transverse deposition and the quantity of external longitudinal microfibrils (see text, Section 3.3.1). Thus, the difference in retardation between the thickenings and the adjacent wall faces is slight.

(b) In uniform field interference, the longitudinal thickenings (e.g., arrows) are visible (cf. Fig. 3.7b) and do not encroach upon the end walls. (a) and (b) x 200.

Figure 4.15:

A complete day 2 cell.

(a) In polarised light, as Figure 4.14a, except that a change in pit shape (e.g., white arrow) is apparent.

(b) In uniform field interference, as Figure 4.14b.

(c) In fringe field interference, the longitudinal bands in the side wall (examples of positions marked by arrows) are, as before wounding (see Fig. 3.7c), thicker than the adjacent faces. (a) x 150; (b) and (c) x 125.

Figure 4.16:

A complete day 3 side wall.

(a) In contrast to Figures 4.14a and 4.15a, the longitudinal bands are no longer visible in polarised light. Indeed, it can be seen that two areas of wall (asterisks) have near neutral retardation and circular pits (e.g., arrow); the other two portions (stars) have also changed in appearance, but to a lesser extent (end walls not shown).

(b) In uniform field interference, the longitudinal bands, which are not visible in polarised light (a), are apparent (arrows). A portion of an end wall (E) impinges on the edge of the image.

(c) In fringe field interference, the longitudinal bands (examples of positions marked by arrows) are still thicker than the adjacent wall faces, despite the variation in deposition across the wall that is apparent in (a). (a) x 300; (b) and (c) x 200.
Figures 4.17 - 4.20:

Figure 4.17:

Day 4 pith cell isolated by EGTA extraction and cut open.

(a) In polarised light, as observed in Figure 4.16a, the wall has changed markedly in appearance from the unwounded condition (see Fig. 3.7a), but this is more prominent in two areas of wall (asterisks). The areas of greater change equate with the radial walls and those of lesser change with the periclinal walls (see text, Section 4.3.1). Many areas of wall now exhibit longitudinal order overall and the pits (e.g., arrow) show Maltese Crosses. End-wall (e.g., E) pits also show signs of circumferential reinforcement (e.g., arrowhead).

(b) In sharp contrast to the unwounded condition (see Fig. 3.7b), the picture in uniform field interference is markedly different from that in polarised light (a). The longitudinal bands (e.g., arrows) remain prominent despite their obliteration in polarised light images. The same specimen, but as seen in white light, where a difference is optical thickness is manifested by a change in colour, is presented as the frontispiece (Figure 1.1).

(c) In fringe field interference, with the fringes running down the length of the side wall, it can be seen, by the straightness of the displacement, that the differences in thickness around the cell in (d) are manifested across the width of the wall and not down its length. (d) In fringe field interference, the substantial increase in thickness that has occurred after wounding is shown by the greater fringe deflection than in unwounded samples (see Figure 3.7c). The longitudinal bands (examples of positions marked by arrows) are still thicker than the adjacent cell-faces. (a) x 265; (c) x 135; (b) and (d) x 185.

Figures 4.18 - 4.20:

End walls.

Figure 4.18:

Transverse hand-section from a day 2 wound as seen in polarised light.

(a) Prior to further trimming; several end walls, which lack overall order, are visible in focus flat against the slide (e.g., arrow). W; wound.

(b) After trimming; the arrowed wall from (a) and its neighbours have little order and no prominent Maltese Crosses over the pits (example of position marked by arrow). The bright lines (asterisk) are end-on views of pieces of attached side wall. (a) x 80; (b) x 255.

Figure 4.19:

Transverse hand-section from a day 4 wound as seen in polarised light.

(a) Prior to further trimming; several end walls lie against the slide (e.g., arrow), although there has been no switch to overall order the end walls are not as vapid as unwounded (see Fig. 3.17), or even day 2 (Fig. 4.18), specimens. W; wound.

(b) After trimming; one of the walls as seen at higher magnification and a different compensator setting. Not only are Maltese Crosses
prominent over the pits (e.g., arrow), but the wall is also approximately divided into quadrants - two light, two dark. (a) x 80; (b) x 400.

Figure 4.20:

Electron micrograph of a day 4 end wall from a periclinal-longitudinal-section (i.e., cut parallel to the wound surface). Considerable thickening has occurred after wounding (original-wall boundary marked by white truncated-arrows). The new deposition is layered with approximately periclinally- (e.g., white arrows) and anticlinally- (e.g., black arrows) oriented lamellae. X 37,000.
Figures 4.21.- 4.24:

Electron micrographs of the polylamellate deposition laid down after wounding. I. Conventional microscopy.

Figure 4.21:

Grazing radial-longitudinal-section at the junction of an end (E) and radial wall; day 1. The original random-nature of the deposition in the end wall (asterisk; boundary of original wall marked with white truncated- arrows) and the transverse microfibrils of the side wall (e.g., white arrow) can be seen. A layer of periclinal post-wounding deposition (end views of microfibrils) is apparent (e.g., black arrows) on both sides of the end wall, which spills over onto the adjacent radial wall and thus becomes longitudinally-aligned.

In the main cell, longitudinally-oriented microtubules are present (e.g., black arrowhead), although in the other cell the microtubules are more transverse (e.g., white arrowhead), perhaps indicating the direction (anticlinal) of the second layer of post-wounding deposition. x 30,000.

Figure 4.22:

Day 1; transverse section of a radial wall from the same cell (right-hand portion of the apposed wall) as used in Figure 4.7. The original transverse deposition can be seen as lines (white star). Also visible are the post-wounding longitudinal microfibrils (black arrows); the boundary between the pre- and post-wounding deposition is marked by white truncated-arrows. x 30,000.

Figure 4.23:

Grazing radial-longitudinal-section of a radial wall; day 1. The original transverse deposition (e.g., white arrow) and the longitudinal microfibrils laid down after wounding (e.g., black arrow) are apparent, as are transverse microtubules (white arrowheads). These are presumably about to participate in the deposition of second (transversely-aligned) post-wounding layers of microfibrils. x 30,000.

Figure 4.24:

Day 4; transverse section of a radial wall from the same cell (left-hand portion of the apposed wall) used in Figures 4.7 and 4.33. As Figure 4.22, except that several alternating layers of longitudinally- (black arrows) and transversely- (white arrows) aligned microfibrils are present. The pattern of deposition is mirrored to a large extent in the two apposed cells. x 30,000.
Figures 4.25 - 4.26:

Electron micrographs of the polylamellate deposition laid down after wounding. II. Stereo microscopy (but not presented as stereo pairs for stereo viewing). Walls from cells responding to wounding were examined at angles of tilt from 45°C to +45°. Sections were viewed with the axis of tilt both at 90°C to and parallel with the middle lamella. In none of the many samples examined were helicoidal transitions (reversible parabolic arcs) observed. In the examples presented here the axis of tilt runs parallel with the middle lamella (i.e., across the page in Figure 4.26 and up and down it in Figure 4.25). All figures are x 50,000.

Figure 4.25:

Day 4; transverse section of a radial wall; (a) +30°; (b) 0°; (c) -30°.

As in Figure 4.25, microfibrils are arranged similarly within each layer as demonstrated by the longitudinally-aligned layers (black arrows) changing from dots in (a) to short lines in (b) and (c). Again, the boundary between transverse (white arrows) and longitudinal layers is abrupt and the former appear to be in the same orientation as the original pre-wounding deposition (e.g., star; boundary of pre- and post-wound wall material marked by the white truncated-arrow). However, in some of the later layers to be deposited after wounding, an intermediate (45°C) layer appears to be visible (e.g., white arrowhead). The herringbone pattern that is apparent at these sites is a feature of cross-ply walls that have been sectioned obliquely (see Chafe and Doohan, 1972) and so 45°C layers may or may not be present. Regardless of this, major changes in the orientation of the microfibrils in successive layers still occur.

Figure 4.26:

Day 2; longitudinal section of a radial wall; (a) -45°; (b) 0°; (c) +45°.

The transversely-aligned layers (white arrows) appear as dots in (a) and they become short-lines in unison as the section is tilted - (b) and (c). This indicates that the microfibrils within the two marked lamellae are aligned parallel to each other (i.e., no gradual changes in orientation). Furthermore, the boundary of these transverse layers and the longitudinal layer between them (black arrows) is abrupt. Note how the early layers of post-wounding deposition visible in (a) (black arrowhead) are lost from view as the section is tilted (c).
Figures 4.27 - 4.31:

Electron micrographs of the polylamellate deposition laid down after wounding. III. Continuity of lamellae.

Figure 4.27:

Day 4; transverse section of the wall by an intercellular space at a radial (R)/outer-periclinal (OP) wall junction (the upper of the two OP/R corners in Figure 4.33). Beyond the original transverse deposition (star; boundary of the pre- and post-wounding deposition marked by the white truncated-arrow), alternating longitudinal (e.g., black arrow) and transverse (e.g., white arrows) layers can be seen. These are continuous around the corner. x 22,000.

Figure 4.28:

Day 2; grazing longitudinal-radial-section of a radial wall near an intercellular space. Major switches in the direction of cellulose microfibril orientation between the post-wounding layers is apparent (e.g., arrows). Microtubules (e.g., arrowhead), approximately transversely aligned, are also present. x 40,000.

Figure 4.29:

Day 4; longitudinal section cut approximately parallel with the wound surface at the site of a radial- (R) and transverse-end- (E) wall junction. Post-wounding lamellae (e.g., arrows) are apparent which are continuous around the corner. x 40,000.

Figure 4.30:

Day 2; transverse section of a radial wall from a plant grown in a constant environment. Longitudinal (e.g., black arrows) and transverse- (e.g., white arrow) lamellae, similar in appearance to those laid down after wounding in plants grown in a changing environment, are present outside a typically transverse-aligned pre-wounding wall (star; boundary marked by white truncated arrows). x 40,000.

Figure 4.31:

Day 4; periclinal longitudinal section at the site of a pit (P) in a thickened end-wall. Lamellae (e.g., arrow) typical of suberin are present on both sides of the wall. x 40,000.
Figures 4.32 - 4.34:

Diagrams of cortical microtubule distribution (microtubule diameters not to scale) in cells responding to wounding, as seen in transverse section. Microtubules were scored, from micrographs of the total perimeter of the cells, as transverse (shown as lines), longitudinal (shown as dots) or oblique (shown as oblique lines) as per Hardham et al. (1980). Particular care was taken to mark obliquely-oriented microtubules as such, in view of the postulation that polylamellate walls may be laid down helicoidally under the guidance of microtubules (see Lloyd, 1984a). All figures was positioned as if the wound was up and down the left-hand side of the page; IP, inner periclinal-; OP, outer periclinal-; R, radial-wall; N, nucleus.

Figure 4.32:

Day 1; the nucleus of the cell has exhibited traumatotaxis and has some microtubules associated with it, which may explain the mixed orientation of microtubules in that region. Otherwise, the periclinal walls generally possess transversely-aligned microtubules whilst those against the radial walls are mainly in a longitudinal orientation. Some post-wounding deposition has taken place around all the side walls of the cell.

Figure 4.33:

Day 4; the nucleus of the cell is in a systrophe (determined by serial sectioning) and an adjacent cell has recently divided. In addition, the walls have considerably thickened (Figs. 4.8, 4.24 and 4.27 are taken from this cell). Periclinal walls possess transversely- and radial walls longitudinally-oriented microtubules, but the microtubule layout is much more clear cut than in Figure 4.32. Not only has the density of microtubules increased, but each wall face is nearly uniform in terms of the alignment of the microtubules against it.

Figure 4.34:

Day 5; considerable wall thickening has occurred and the neighbouring cells have recently divided in a periclinal direction. The pattern of microtubule distribution is very much as in Figure 4.33, except that the orientation is reversed; periclinal walls possess longitudinally- and radial walls transversely-aligned microtubules.
DAY 1

DAY 4

DAY 5
Figures 4.35 - 4.43:

Transverse-wound response. All figures are oriented as if the wound was across the top of the page.

Figures 4.35- 4.40:

Longitudinal hand-sections examined using DIC optics.

Figure 4.36:

Day 1; an organelle-enclustered nucleus can be seen (arrow) against a longitudinal wall. x 190.

Figure 4.36:

Day 3; three organelle-enclustered nuclei (black arrows) are present, all with cytoplasmic strands (white arrows) radiating from them. That this early stage in the formation of systrophes does not occur from the apical end-wall (the wound wall in this type of wound) demonstrates that traumatotaxis does not occur (cf. longitudinal wounds, see Section 2.3.5). x 190.

Figure 4.37:

Day 3; a systrophe (arrow) seemingly identical in appearance to those formed in response to longitudinal wounding (e.g., see Fig.2.22). x 190.

Figure 4.38:

Day 4. (a) View of two cell-plates (arrows) forming transversely, i.e., parallel to the wound surface. x 190. (b) Higher magnifications of one of the cell plates (arrow) from (a). x 475.

Figure 4.39:

Day 4; new walls may (black arrowhead) or may not (white arrowhead) be lined-up in 4-way junctions. x 190.

Figure 4.40:

Day 4; subsequent rounds of division follow a similar pattern to the initial one. Two systrophes (arrows) are apparent on either side of a new wall (arrowhead). x 190.

Figures 4.41 - 4.43:

Longitudinal hand-sections examined in polarised light; W, wound. All x 135.

Figure 4.41:

Day 3; longitudinal walls (e.g., arrow), from the cells that are undergoing a cytoplasmic wound-response, are unchanged in appearance from the unwounded state (e.g., see Figure 3.2a).

Figure 4.42:

Day 5; longitudinal walls immediately under the wound (arrowhead) and those away from it have a similar (unchanged) appearance. Thus, no extra cellulose has been deposited in response to wounding. This is in marked
contrast to the longitudinal-wound response (cf. Fig. 4.13).

Figure 4.43:

Day 7; cells under the wound have divided several times and their longitudinal walls have expanded and have a retardation close to zero (star). On the other hand, undivided cells (arrow), immediately under the meristematic tier, retain their pre-wounding appearance.
Diagrammatic representation of the postulated mode of cellulose deposition after wounding.

The images in column O represent the pre-wounding condition, whilst columns 1, 2 and 3 illustrate the successive initial stages of the wound response. In each column, (a) shows the cortical microtubule distribution throughout the cell, (b) gives a cross-section through the longitudinal walls of the cell showing both the cortical microtubule distribution (dashes and circles) and the microfibrillar pattern (lines and dots) and (c) illustrates the microfibrillar pattern in the end walls as seen in surface view.

0. Before wounding, the side walls possess both transverse microtubules (a and b) and cellulosic reinforcement (b). The end walls have straight, randomly-oriented microtubules (a) as well as a similar lack of overall cellulosic order (c).

1. After wounding, a longitudinal hoop of cortical microtubules forms, which encompasses both the end and two of the side walls of the cell (a) (in this illustration, assuming the wound to have been up and down the left-hand side of the figure, the hoop initially lies under the periclinal walls). This results in a layer of longitudinal deposition in these periclinal walls. The microtubules lining the other two side walls (the radial walls in this example) remain transverse (a and b) and so the deposition also remains transverse and cannot be distinguished from the wall material laid down before wounding (b). In the end walls, a layer of ordered (anticlinal) deposition occurs, as a result of the continuity of the hoop.

2. The longitudinal hoop changes position to encompass the other two side walls, in this example the radial walls. The microtubules underlying the longitudinal walls no longer contained by the hoop, i.e., the periclinal walls, revert to the stable 'memory position' (after Green [1984] - see text, Section 4.4) of transverse orientation (a). This results in transverse microfibrils being laid down interior to the initial, longitudinally-oriented, post-wounding layer. Thus, the characteristic polylamellation of the novel deposition is initiated (b). Simultaneously, the radial walls deposit longitudinal microfibrils, which generate the first post-wounding layer distinguishable in these walls. The continuity of the hoop results in a second ordered layer of deposition in the end walls (c). This cancels out the initial anticlinally-oriented layer and so maintains the neutral birefringence of the end walls when seen in polarised light (e.g., see Fig. 4.18a).

3. The longitudinal hoop switches position again to lie in its original post-wounding configuration. Longitudinal walls not encompassed by the hoop revert to a transverse orientation (a). This continues the development of the polylamellate construction in the longitudinal walls (b). Simultaneously, another layer is deposited on the end walls and so they too develop a polylamellate pattern.

The switching of the position of the longitudinal hoop of cortical microtubules continues, until the onset of mitosis, thus building up a polylamellate wall against all faces of the cell. Differences in the thickness of the layers on different walls could be achieved through changes in microtubule density (not illustrated).
CHAPTER FIVE

IMMUNOCYTOCHEMICAL LOCALISATION OF ACTIN AND TUBULIN IN THE WOUND RESPONSE

The study of the role of cortical microtubules in regulating the movement of plant cells has been useful in elucidating the mechanism of growth and differentiation (Raghavan, 1975). Recent advances in technology, particularly with regard to antibodies and immunochemistry, have enabled the application of anti-tubulin antibodies for immunocytochemical studies in many plant tissues. This has facilitated the study of the organization, pre-assembly, spindle and segregation, and other aspects of mitosis and cytokinesis (Clayton and Lloyd, 1983; Taylor et al., 1981; Nickell et al., 1981; Nickell and Gurley, 1982, 1983), the origins of microtubule organizing centers and division planes (Clayton and Lloyd, 1983; Taylor et al., 1981; Nickell et al., 1985), as well as the potential role of microtubules in the control of cellulose deposition (Lloyd, 1983) and cell wall deposition (Taylor et al., 1985).
5.1 Introduction

Immunocytochemical techniques have been used to locate cytoskeletal elements and study their roles in many types of animal cell (e.g., see Lazarides, 1982; Osborn and Weber, 1982). However, in plants the application of such techniques has been relatively restricted, mainly owing to the paucity of suitable antibodies and the difficulty of applying them to walled cells. The conserved nature of cytoskeletal proteins has enabled some antibodies raised against animal antigens to be used successfully on plant tissue (see below). More recently, monoclonal antibody (MAB) technology (Fazekas de St. Groth and Scheidegger, 1980) has not only reduced the need to purify antigens, but has also provided large amounts of antibodies. A number of MABs, raised against animal and yeast tubulins, but reactive with plant cytoskeletal components, is now available.

The other main problem in immunocytochemical studies of the cytoskeleton in plants has been that of the cell wall, whose small pore size (Carpita et al., 1979) prevents the passage of antibodies. In some cases the use of specialised naked cells has circumvented this problem, as in the study of the role of cortical microtubules in maintaining the shape of protoplasts (Lloyd et al., 1979a,b; 1980). The technique of wall degradation and tissue squashing introduced by Wick et al., (1981) has enabled the application of anti-tubulin antibodies, for immunofluorescence, to walled plant tissues. This has facilitated the study of interphase, pre-prophase, spindle and phragmoplast microtubules (Wehland et al., 1984; Wick et al., 1981); the transitions between these arrays (Wick, 1985a; Wick and Duniec, 1983, 1984); the effects of drug treatments upon mitosis and division planes (Clayton and Lloyd, 1984; Tiwari et al., 1984; Wick, et al., 1985), as well as the putative role of microtubules in the control of cellulose deposition (Lloyd, 1983) and cell morphogenesis (Traas et al.,
1984). Recently, these studies have been extended to the use of anti-tubulin antibodies on sections of polyethylene glycol-embedded material (Gunning and Wick, 1985; Tiwari et al., 1984; Wick, 1985a) thus permitting retention of the relative positions of the cells within the tissue (which are usually lost as a result of the squashing technique).

The localisation of F-actin within plant cells has been more limited than it has for tubulin, most studies to date having been done on algae. Immunofluorescence with a specific human autoantibody and an antibody to Physarum actin demonstrated that subcortical bundles of Chara (Williamson and Toh, 1979) and Nitella (Owaribe et al., 1979) contained F-actin, confirming other identifications made by means of labelling with heavy meromyosin in respect of Chara (Williamson, 1974), Nitella (Palevitz and Hepler, 1975; Palevitz et al., 1974) and other algal species (Blatt et al., 1980; Klein et al., 1980; Marchant, 1976). In higher plants the same technique has been used in Amaryllis (Condeelis, 1974), Haemanthus (Forer and Jackson, 1979) and in radish root hairs (Seagull and Heath, 1979) whilst, at the level of the light microscope, cytoplasmic strands in conifer roots (Pesacreta et al., 1982) and Tradescantia staminal hairs (Gunning and Wick, 1985; Tiwari et al., 1984) have been shown to contain F-actin by the use of fluorescently-labelled specific phallotoxins. MABs against plant actin have only recently been raised (Tiwari et al., 1984; Williamson et al., 1984), but their utilisation to demonstrate the presence of actin at the periphery of chloroplasts by McCurdy (1983) is, to date, the only report of the use of anti-actin antibodies of any sort on higher plants.

In the work described in this chapter, immunolocalisation has been used to investigate the distribution of F-actin during post-wounding nuclear migration, and the relative orientation of cortical microtubules
and cellulose microfibrils. In both studies, one general advantage of immunofluorescent techniques was of particular value: the combination of high sensitivity (see Brinkley et al., 1980) with the facility to examine large areas to gain a picture at the level of the whole cell.

Drug studies on traumatotaxis in *Tradescantia* leaves (Schnepf and von Traitteur, 1973) and in the formation of systrophes in a number of species (Weidinger, 1980a) (detailed in Chapter 2) have suggested that F-actin is involved. The investigative procedure adopted in the present work relied upon examination of cut-open cells in hand sections of paraformaldehyde-fixed tissue. The technique was akin to that carried out using cut-open *Chara* cells when originally selecting the MAB-producing hybridomas (McCurdy, 1983; Tiwari et al., 1984; Williamson et al., 1984). Cut cells in thick sections (cut whilst frozen and then fixed) of *D. stramonium* stems had previously been used in immunofluorescence studies (Jeffree and Yeoman, 1981) of seed lectin of the same species (Kilpatrick and Yeoman, 1978; Kilpatrick et al., 1979). After many refinements, reasonable preservation of decollated cells was obtained in the experiments described in a portion of this chapter, and the MABs used were shown by preabsorption to be localising F-actin. The data obtained were supplemented by the use of the F-actin specific probe NBD-phallacidin (Barak et al., 1980, 1981), by means of which uncut cells could be examined.

Also described herein are attempts, both to extend the anti-actin work to the level of the electron microscope by the use of the pre-embedding colloidal gold-labelling technique (see Roth, 1983) and to further develop the procedure to allow post-embedding of methacrylate-sectioned material—as has been done by Howlett et al. (1981) with pollen allergens.

The remaining portion of this chapter describes the development and use of immunofluorescence experiments to reveal the distribution of
cortical microtubules during the wound response, with especial regard to their role in the control of wall deposition. In order to investigate such a relationship it was apparent that existing methods that involved wall degradation (Wick et al., 1981) would be inappropriate and so a novel protocol had to be developed. This was based on the technique of cutting open EGTA-isolated fixed cells, as used for polarisation and interference studies of wall changes (Chapters 3 and 4), and the use of the detergent Triton-X 100 (T-X) in the fixative solution, in order to permeabilise the cytoplasm sufficiently to allow antibody penetration. This procedure allowed visualisation of cortical microtubules and the determination of adjacent wall characteristics in the same specimen.
5.2 Materials and Methods

5.2.1 Application of antibodies to hand sections

5.2.1.1 Protocols

In the batch of plants used for these experiments, the first round of division was generally under way by day 3. Plants of this age were utilised as cells at most stages of the cytoplasmic wound response were present in single sections.

Hand sections of varying thicknesses were cut, from the central portion of the wound, under fixative comprising 2.5% (w/v) paraformaldehyde in buffer (25mM potassium phosphate) and fixed for 1h. As antibodies are large molecules (mouse immunoglobulin G [IgG] is around 150,000 daltons, Hudson and Hay, 1980) they cannot pass through plant cell walls or intact membranes and so can only penetrate cells that have been actually cut open in such sections (see Knox, 1982a). The ideal section thus encompassed a high proportion of cells with one end wall cleanly removed and the bulk of the cytoplasm left intact. In individual experiments at least four sections were subjected to each treatment, and where more than one plant was used, sections were apportioned equally among the various treatments. After cutting, one or two sections from each plant were examined in order to determine the stage of the wound response. Fixed sections were rinsed 3 x 7 min in buffer and gently placed onto microscope slides using a fine paint-brush. Each section was incubated in 10µl of primary antibody for 2h in a dark, moist environment at either 37°C or RT. Sections were rinsed, the secondary antibody (the conjugate) applied for 30min, rinsed again and mounted in buffer under "Valap" sealed coverslips. Desiccation, which caused cytoplasmic collapse, was avoided throughout the above protocol and in the modified scheme used in later experiments, which made use of flat-bottomed micro-titre plates (Nunc, Denmark). In the altered protocol,
after fixation, sections were transferred, one per well, to the plates, where all subsequent processing was carried out. This reduced handling (and therefore damage) of the sections, which were examined using a Nikon Diaphot inverted microscope equipped with phase contrast optics, prior to application of the antibodies, thus permitting the best examples to be selected. In all individual experiments, controls were carried out by using buffer in place of the appropriate antibody, checking for autofluorescence and non-specific binding of the second antibody.

5.2.1.2 Antibodies and phallotoxin

The primary antibodies mainly used were those MABs designated CC2, CC4 and CC5, that had been raised and generously supplied by Dr. R.E. Williamson and colleagues (R.S.B.S., A.N.U.). They were prepared by raising MABs from mice (see Fazekas de St. Groth and Scheidegger, 1980) against a crude preparation of cortical cytoplasm expelled from internodal cells of Chara corallina and selecting by immunofluorescence those which became bound to the subcortical actin bundles. Briefly, all three clones bind strongly to these fibres and binding to other sites is extremely limited except that CC2 also binds to transversely-oriented fibrils beneath the plasmalemma (Tiwari et al., 1984; Williamson et al., 1984) and CC4 binds to dictyosomes (Craig, 1984). All MABs were used undiluted as supernatant fractions that had been precipitated with ammonium sulphate, resuspended and dialysed overnight at 4°C against perfusion solution (PS, made up as in Williamson et al., 1984) or phosphate-buffered saline (PBS). CC4 was mostly used as it was the most fully characterised (see McCurdy, 1983, where it was designated UEA2 D2).

A polyclonal primary antibody raised against porcine brain tubulin (kindly supplied by Dr. S. Wick, R.S.B.S., A.N.U.) was also used, in a
modified protocol. Fixation was carried out in 3.7% (w/v) paraformaldehyde plus 5mM EGTA, rinsing was in PBS and the antibody was diluted 1+3 with buffer prior to use.

Fluorescein (FITC)-conjugated rabbit anti-mouse IgG (RAM-FITC; Miles-Yeda, Israel) diluted 1+19 with phosphate buffer was usually employed as the conjugate, although rhodamine (TRITC)-conjugated rabbit anti-mouse IgG (RAM-TRITC; Miles-Yeda) diluted 1+9 with buffer was also used. However, the author is insensitive to the wavelengths of light that TRITC emits and so it could only be used in conjunction with a video system (see Section 5.2.1.3).

To determine general binding properties of mouse immunoglobulin (Ig) to sites in D. stramonium pith cells, an irrelevant MAB, designated ACR, was used as the primary antibody. This was raised against acetylcholine receptors from Torpedo marmorata (see Watters and Maelicke, 1983) and was kindly provided as a culture supernatant by Drs. C. Hill and D. Watters (J.C.S.M.R., A.N.U.).

In some cases, ACR and CC4 were absorbed overnight at 40°C with F-actin prior to use, at 4x and 40x the estimated Ig concentration of CC4, according to the technique of McCurdy (1983). Bovine serum albumin (BSA; Sigma, PO Box 14508, St. Louis, Mo, 63178, U.S.A.) was added to the lower concentration and to a control to such an extent, that the same amount of extra protein was added to the MABs so that the dilution factor was identical in both.

BSA at 1% (w/v) in buffer followed by a buffer rinse, was also used as a pre-treatment to block protein binding sites within the cells after the post-fixation rinse. When CC4 and ACR were the primary antibodies, equal molarities of NN'-diacetylchitobiose and NN'N''-triacetylchitobiose (both Sigma) in buffer at 5 or 50mM total saccharide concentration were sometimes
used as a pre-treatment (in the controls, buffer only) after the BSA. In such cases, these mixed oligomers of N-acetylglucosamine, which strongly inhibit the activity of the lectin that is found in *D. stramonium* (Kilpatrick and Yeoman, 1978), were also added to the MABs (to the controls, equal volumes of buffer only were added).

NBD-phallacidin (7-nitrobenz-2-oxa-1,3-diazolylphallacidin; NBD-Ph) which is supplied in methanol (Molecular Probes, Inc., Junction City, Or, 97448, U.S.A.) was dried under nitrogen and taken up in a half volume of buffer prior to use. The usual control utilised was NBD-Ph with a 50x excess of phalloidin (NBD-Ph+Pd; phalloidin (Pd) supplied by Molecular Probes), although in some cases sections were pre-treated with Pd in buffer for 20 min at RT prior to the application of NBD-Ph. The protocol was similar to that used for the antibodies (above). After fixation and rinsing, incubation was for 1h at RT prior to rinsing and examination using the same filters as for FITC and, because of the low level of fluorescence, the video-enhancement system (see Section 5.2.1.3).

5.2.1.3. Microscopy

Specimens were examined using epifluorescence optics on a Zeiss Photomicroscope III fitted with a CSI 250w lamp and filters standard for FITC and TRITC. A KP600 barrier filter was also used with the former to eliminate the red chloroplast-autofluorescence. Micrographs were made on Kodak Tri-X rated at 1600 ASA (DIN 33), which was developed in Diafine (Acufine Inc., Chicago, Il, U.S.A.). Exposure times for the CC MABs were those given by the automatic spot meter built into the microscope ('automatic' exposures). These times were recorded and the maximum used for the controls in each individual experiment (maxima were very similar in different experiments) in order to show quantitative differences in the
level of fluorescence ('timed' exposures). In order to illustrate qualitative differences the automatic facility was also used. 'Dry' phase objectives were usually employed as these obviated the problems of specimen movement when using oil immersion on the relatively thick sections.

In later experiments a video system was used to enhance and record the images, especially those of low intensity. The output of a Silicon Intensified Target video camera (DAGE-MTI Inc., Michigan City, In, U.S.A.) was fed through a video processor (Model 604, Colorado Video Inc., Boulder, Co, U.S.A.), a time-date generator (VTG-33, FOR-A Co Ltd., Tokyo, Japan) and a temporal filter (TF 4000, Arlunya, P.O. Box 106, Vermont, Vic. 3133, Australia) to a 3/4 inch video cassette recorder (Sony V-5800PS). Photographs were taken from the monitor using a Kodak Instagraphic CRT Print Imager.

5.2.2 Application of antibodies to embedded material

5.2.2.1 Pre-embedding labelling for electron microscopy

Hand sections from day 2 or day 3 plants were taken through the standard protocol (Section 5.2.1.1), using CC4 or buffer as the primary antibody, to the second antibody stage. Sections were then either processed for immunofluorescence or, for EM, treated for 30min at RT with goat anti-mouse IgG coupled to 20nm colloidal gold (GAM 20; Janssen Pharmaceutica, Belgium) diluted 1+4 with buffer. This gave a 'treatment' and a 'gold-only' control (from those sections treated with buffer rather than CC4). After rinsing, these samples for EM were re-fixed in standard EM fixative (Section 2.2.5) for 2h at RT, rinsed and post-fixed in 1% (w/v) OsO₄ in buffer for 20 min prior to processing through the remainder of the standard EM protocol (Section 2.2.5). Longitudinal and transverse sections were cut and stained for 7 min in uranyl acetate and 2 min in lead citrate prior to electron microscopic examination.
5.2.2.2 Post-embedding labelling (of Chara corollina) for light microscopy

Internodal cells of C. corollina were cut into segments approximately 3mm long and swirled gently for 5 min in PS (McCurdy, 1983) prior to fixation in either 1% (w/v) paraformaldehyde in PS for 20 min or 2% (w/v) for 20 or 40 min. After 3 x 10 min rinses in PS (used for all subsequent washes), some segments were processed for standard immunofluorescence using CC4 (Williamson et al., 1984), whilst others were dehydrated directly, infiltrated for 48h and embedded in SM medium (Section 2.2.3.2) at 40C. 2 µm sections were cut (Section 2.2.3.2), air-dried onto slides and incubated, either directly or following a 15 min pre-treatment with 1% (w/v) BSA in PS and a 10 min rinse, for 30 min at RT with CC2, CC4 or CC5. After a 15 min rinse, sections were treated for 30 min with RAM-FITC (1+19 in PS) or, for the autofluorescence control, with PS prior to rinsing and mounting in PS or water for microscopy as per Section 5.2.1.3.

5.2.3 Application of antibodies to opened-out cells

5.2.3.1 Protocols

In initial experiments, hand sections were cut from unwounded and days 1, 2 and 3 plants (as in Section 2.2.3.1) and fixed for 2h in 2.4% (w/v) paraformaldehyde or 2.5% (v/v) glutaraldehyde, in either buffer or PBS, at RT. After rinsing (3 x 20min), samples were left overnight, with one change of solution, at 40C in buffer or 50mM EGTA in buffer or water prior to 2h in fresh EGTA in water at RT and squashing in the same solution. All subsequent manipulations were carried out with the aid of a dissecting microscope. Files or individual cells were picked out of the suspension using the tip of a micro-knife and placed into a drop of PBS or EGTA solution on 13mm diameter coverslips (Chance Propper Ltd., Smethwick,
Warley, England). These had been thoroughly cleaned, by sonication in acetone, and coated with 0.1% (w/v) high molecular weight (520,000 daltons) poly-L-lysine (PLL; Sigma, PO Box 14508, St. Louis, Mo, 63178, U.S.A.), as recommended by Huang et al. (1983). After partial drying with a piece of filter paper, cells were cut with a razor blade across the side walls adjacent to the end walls and lengthwise down the side wall prior to rewetting and the gentle opening out of the cell in a manner similar to that described in Section 3.2.2. Care was taken to disturb the cellular contents as little as possible and the wetting/drying cycle of the standard carving technique was avoided. This reduced considerably the time required, but meant that cell dissection was not as completely carried out. Upon opening, walls stuck tenaciously to the PLL and their position was subsequently marked on the reverse side of the coverslip with a diamond-tipped pen. After carving, all the cells on one coverslip (usually 2 or 3 single cells or one file) were gently blotted (carried out after all subsequent rinses) and incubated with 5 µl of primary antibody (see Section 5.2.3.2) for 1-2h (carving was carried out for 1h) at 37°C in a humid atmosphere in the dark. All coverslips were then rinsed (3 x 3 min) in PBS, incubated with 7.5 µl of conjugate (see Section 5.2.3.2), rinsed again and mounted in Mowiol 4-88 (Hoechst, Frankfurt, F.R.G.) medium (Osborn and Weber, 1982). This is glycerol-based and so is water miscible and contains n-propyl gallate to reduce the fading of the fluorescence (Giloh and Sedat, 1982). Slides were stored at 4°C in the dark prior to examination as described in Section 5.2.3.3. In some cases samples were briefly air-dried prior to the application of the primary antibody, but otherwise drying was avoided at all times. The above protocol was modified in later experiments, initially by the use of 1% (v/v) T-X for 10 min or methanol at 0°C for 2 min after cutting open the cells and prior to antibody
application. Later, following the use of T-X in experiments for EM, (Section 5.2.4), tissue was fixed for 2h in 3% (w/v) fresh, filtered paraformaldehyde in microtubule-stabilising buffer (see Section 5.2.4), with or without 0.5% (v/v) T-X. After fixation, samples were rinsed 5 or 6 times in 2h in 50mM EGTA in water prior to storage overnight at 4°C in 50mM EGTA plus 0.3mM PMSF (Sigma, P.O. Box 14508, St. Louis, Mo, 63178, U.S.A.) and subsequent processing as described above.

5.2.3.2 Antibodies

The primary antibodies used were the MABs YL 1/2, YOL 1/34 (Sera Laboratories, Crawley Down, Sussex, U.K.), TU-01 (Transakata Pzo, Praha, Czechoslovakia) and K2D7B8 (kindly provided by Dr. K. Mizuno and Ms J. Perkin, R.S.B.S., A.N.U.). The 'Kilmartin' MABs, YL 1/2 and YOL 1/34, had been raised against yeast α-tubulin (Kilmartin et al., 1982) and were supernatant fractions used at 1+9 or 1+49 in PBS. Anti-pig brain tubulin TU-01 (Viclický et al., 1982) was used as an ascites fluid (1000µg ml⁻¹ IgG) diluted 1+199 in PBS together with 0.1% (w/v) BSA and 0.02% (w/v) sodium azide. Anti-mung bean α-tubulin K2D7B8 (Mizuno et al., 1985b) was used as an undiluted supernatant or as an ascites fluid diluted 1+5 or 1+9 with PBS.

In some experiments 1% (w/v) BSA was used as a pre-treatment at 37°C or RT for 20 to 40 min prior to antibody application. Absorption of TU-01 with pure mung bean tubulin (a gift of Dr. K. Mizuno, prepared as in Mizuno, 1985) at 10x and 50x IgG concentration was also carried out, using BSA as a control in the same manner as for the actin pre-absorption (Section 5.2.1.2). RAM-FITC, diluted 1+19 with PBS was used as the conjugate.
5.2.3.3 Microscopy

Fluorescence microscopy was carried out as described in Section 5.2.1.3 except that, because of the nature of the specimens, high numerical aperture oil immersion lenses could be used.

After immunofluorescent observation of the cytoplasm, the properties of the walls were examined using polarising (Section 3.2.3) and interference optics (Section 3.2.4). However, when carried out in situ, crystals of PBS and other refractive or birefringent objects reduced the quality of the polarised light image. Also, the high refractive index of the Mowiol (the n of glycerol is approximately 1.47) made visualisation of the specimens difficult in interference images as \( n_{\text{object}} - n_{\text{medium}} \), and therefore OPD, was low. Thus, coverslips were removed by soaking overnight in water and the cells gently prised off in a drop of water using a microknife and transferred to clean slides.

5.2.4. Electron microscopic study of cells preserved in fixative containing Triton-X

Individual days 2 or 3 wounds were cut into transverse sections in microtubule-stabilising buffer (MSB) comprising 100mM piperazine-N,N'-bis (2-ethane sulphonic acid) (PIPES; Sigma Chemical Co), 2mM EGTA and 1mM MgCl\(_2\) at pH7.0 (modification of Luftig et al., 1977).

Immediately after cutting, the sections were divided amongst solutions composed of either 3% (w/v) fresh, 0.22 μm membrane-filtered (Millipore Corp., Bedford, Mass., 01730, U.S.A.), paraformaldehyde in MSB plus 0, 0.2, 1 or 5% (v/v) T-X for 2h 10 min or 1% T-X in MSB for 10min followed by 3% paraformaldehyde plus 1% T-X in MSB for 2h. All sections were further dissected to cubes once transferred. After fixation, specimens were rinsed 3 x 30 min in MSB and post-fixed for 1h in 2% (w/v) OsO\(_4\) in water, prior to subsequent standard processing for EM (Section 2.2.5).
5.3 RESULTS

Unless otherwise stated, all the following descriptions of antibody staining refer to those obtained using RAM-FITC as the secondary antibody.

5.3.1 Localisation of actin

All observations were made on pith cells lacking the autofluorescent suberised walls found immediately under the wound. Using FITC wavelengths, cytoplasmic autofluorescence in 'timed' exposures was very low (Figs. 5.1a, b) and was not detectably structured even in 'automatic' exposures (Figs. 5.2a, b). Lengthy processing increased its intensity (cf. Hardham, 1985) so that freshly-cut sections and NBD-Ph controls were even dimmer. This increase was not alleviated by excluding light as much as possible during processing (cf. O'Brien and McCully, 1981). Nuclear autofluorescence was highly variable, but could be fairly bright (Figs. 5.3a, b). The use of TRITC wavelengths reduced most autofluorescence, but the gain was outweighed by the impossibility of distinguishing chloroplast autofluorescence from a TRITC signal. Except for occasional contamination by brilliantly fluorescent aggregates, neither RAM-FITC nor RAM-TRITC alone markedly altered either the form (Figs. 5.4a, b) or the intensity (Figs. 5.5a, b) of the cytoplasmic fluorescence. The use of a BSA pre-treatment had little or no effect on conjugate- and auto-fluorescence.

The irrelevant primary antibody ACR caused some increase in both nuclear and cytoplasmic fluorescence, but the intensity remained low (Figs. 5.8a, b) and its distribution (Figs. 5.6a, b) was unrelated to that shown by the antibodies directed against the cytoskeleton. It was not significantly reduced by preabsorbing the antibody with F-actin (Figs. 5.9a, b), BSA or the hapten sugars di- and tri-acetylcithitobiose (Figs. 5.7a, b). Video enhancement of the images obtained did not significantly alter the conclusions drawn from any of the preceding experiments.
Low magnification images reveal the bright staining pattern of CC2 (Figs. 5.10a, b) and CC4 (Figs. 5.11a, b). CC5 (not illustrated) gave similar, but dimmer images to those of CC2 and so was only used to a limited extent. With both CC2 and CC4, fibrillar fluorescence was not seen in all stained cells and, in the case of CC4, was often difficult to visualise owing to the bright punctate fluorescence, which represented dictyosomes (see Craig, 1984). Although not as consistent, the above patterns were of a similar type to that observed when the MABs were used on C. corallina. However, when used on D. stramonium, the antibodies also tended to enhance nuclear fluorescence and, in the case of CC4, to increase slightly cytoplasmic fluorescence, but the latter was much reduced by the BSA pre-treatment (cf. Fig. 5.11a with Fig. 5.12a). The possibility that staining was due to antibody binding to the cytoplasm per se was excluded by samples of cytoplasmic strands with no fluorescence (e.g., Figs. 5.16a, b). The addition of oligomers of n-acetylglucosamine to CC4 had no marked effect on fluorescence (not illustrated), but pre-absorption of the antibody at both levels of F-actin, virtually obliterated cytoplasmic staining (Figs. 5.13a, b and 5.14a, b), although variable nuclear fluorescence remained.

The above results are an indication that the CC MABs were binding to F-actin (see Discussion, Section 5.4). It therefore seems that in many cells actin filaments had not been preserved to the degree which allowed their recognition by the antibodies. Nevertheless, some fibrillar fluorescence was observed and so an insight into the possible role of F-actin during some stages of the wound response was obtained. In order to prevent misinterpretation of the cytoplasmic state of the (cut) cells exhibiting antibody staining, they were always examined using phase contrast as well as fluorescence optics.
Putative F-actin could be seen in association with enclustered but, as yet, non-traumatotactic nuclei (Figs. 5.19a, b). However, whether these filaments are involved in cytoplasmic streaming, chloroplast movement or a prelude to nuclear migration is indeterminable. Traumatotactic nuclei may also have been associated with fluorescent fibres (Figs. 5.10a, b). The next stage of the cytoplasmic wound response - the formation of systrophes - could not be properly observed as such structures inevitably collapsed in cut cells (e.g., Figs. 5.15a, b). F-actin was also implicated in cell plate formation as in Figures 5.17a, b, where fluorescence is confined to the phragmoplast - the pattern of the staining demonstrating that fluorescence from the two daughter nuclei was not responsible for the image. After the first division, nuclei were seen near new cross-walls associated with fluorescent filaments stretching across the vacuole to the opposite wall (Figs. 5.18a, b), showing that F-actin is present in the cytoplasmic strands involved in the formation of systrophes.

Dictyosomes, as revealed by CC4, were fairly evenly distributed throughout the cortical and trans-vacuolar cytoplasm, but with clustering around nuclei (Figs. 5.12a, b; 5.15a, b and 5.16a, b) although nuclear fluorescence sometimes obscured this (Figs. 5.11a, b). NBD-Ph is a relatively small molecule (m.w. = 1,012 daltons, data from supplier) and can pass across cell walls and membranes in formaldehyde-fixed tissue (Pesacreta et al., 1982), thus enabling the examination of intact systrophes in uncut cells. Cytoplasmic fluorescence was very low, even after video enhancement, with autofluorescent (Figs. 5.20a, b), NBD-Ph + Pd (Figs. 5.21a, b) and Pd followed by NBD-Ph (not illustrated) controls. By contrast, in NBD-Ph-treated sections, although showing a slight increase in nuclear and general cytoplasmic fluorescence, strands of moderately bright fluorescence were most apparent. Their distributions matched that of the putative F-actin filaments visualised by the CC MABs. Most importantly,
also demonstrated was the presence of actin filaments in the cytoplasmic strands leading up to the initial star formation (Figs. 5.23a, b), in the radiating arms of systrophes themselves (Figs. 5.24a, b) and in phragmosomes (Figs. 5.22a, b). The level of NBD-Ph fluorescence, compared with that obtained with the antibodies, is reduced not only by the thicker sections employed, but also as a result of the lower binding level of NBD to the fluorochrome (1:1) compared with the 4:1 FITC to IgG molar binding ratio in the batch of RAM-FITC used (data from suppliers). In addition, more than one secondary antibody molecule can bind to each primary antibody molecule (e.g., Knox, 1982b).

Supplies of anti-tubulin were severely restricted at the time that these hand sectioning experiments were carried out so only exploratory experiments were performed. These proved to be inconclusive.

In experiments on pre-embedding labelling for EM, those sections processed for immunofluorescence showed the typical control and CC4 staining patterns as described above. At the level of the electron microscope, there was very little non-specific binding by the gold conjugate-only controls - see also McCurdy (1983) and Craig (1984). Detailed examination of 30 cells, which had been identified in longitudinal section as having been cut-open, revealed only a solitary gold particle (Fig. 5.25). In sections treated with CC4, where label was only found in cut cells, nuclei and the borders of nucleoli were labelled (Fig. 5.26). Notwithstanding reports of the location of actin in animal nuclei (e.g., Clark and Rosenbaum, 1979), this probably reflects the non-specific labelling seen in the light microscope (above) and also indicates that sufficient membrane permeabilisation occurs with the protocol used to ensure antibody entry to intracellular structures - indeed the nuclear envelope can be seen to be ruptured in places (Fig. 5.26). Some label was also found at the periphery of amyloplasts (Fig. 5.27). This may reflect
non-specific binding. However, the binding of CC4 to the edge of permeabilised chloroplasts of *Pisum sativum*, *Spinacia oleracea* and *C. corallina* was prevented by actin pre-absorption (McCurdy, 1983). Dictyosome vesicle labelling, as seen by McCurdy (1983) and Craig (1984) was also observed (Fig. 5.28), although not always in association with stacks (Fig. 5.29). This may reflect; either poor preservation of such structures or, possibly, the effects of fixation on a streaming cytoplasmic network (cf. Mersey and McCully, 1978). Label was also found associated with the plasmolyzed plasma membrane (Fig. 5.30). This could represent non-specific labelling, binding to actin within the membrane itself (cf. Pollard and Korn, 1973) or the collapse of the plasma membrane onto a cortical actin network. The survival of such filaments, unlike microtubules which survived processing (Fig. 5.31), remains debatable as no structures resembling the putative F-actin seen in conventionally-prepared tissue were seen (Fig. 3.16). However, label was observed on wispy structures of indeterminate nature (Figs. 5.32 and 5.33) which could represent poorly-preserved F-actin.

In the experiments on post-embedding labelling, segments of *C. corallina* processed directly for immunofluorescence following all fixation regimes showed staining typical of CC4 (work kindly performed by Ms U.A. Hurley and Dr. R.E. Williamson). However, only weak bundle and no granular fluorescence was observed when segments were embedded and treated after sectioning with the CC MABs. In both treatment and control samples, chloroplast autofluorescence was bright, and had changed colour from red to green and thus could no longer be eliminated by the KP600 filter. The use of a BSA pre-treatment had no effect on any fluorescence patterns. This considerable loss of antigenicity meant that post-embedding labelling of *D. stramonium* using this technique would be most unlikely to succeed and so was not attempted.
5.3.2 Localisation of tubulin

The use of anti-tubulin antibodies on opened-out cells resulted in no fibrillar pattern of staining in the cytoplasm, either attached to the walls or adherent to the adjacent PLL when paraformaldehyde or glutaraldehyde in PBS or phosphate buffer was employed. This was also the case when T-X or methanol was applied after cutting open the cells - even after fixation in paraformaldehyde (glutaraldehyde may prevent the penetration of macromolecular tracers into fixed cells, even when they have been permeabilised, Ohtsuki et al., 1978). This led to the testing, using EM on otherwise conventionally-processed tissue, of paraformaldehyde in MSB with or without T-X as an agent to enhance cytoplasmic permeability.

Cells fixed in paraformaldehyde in MSB without T-X, showed a reasonably good level of cytoplasmic preservation with intact cellular, nuclear and organelle membranes (Fig. 5.34) and microtubules (Fig. 5.35). The addition of 0.2% T-X to the fixative caused the disruption of the plasma membrane as well as tonoplast and organelle membranes (Fig. 5.37) and their disintegration when higher concentrations were used (Figs. 5.39 and 5.42). Plasmolysis and considerable extraction of the cytoplasmic ground substance also occurred with increasing concentrations of T-X (Figs. 5.36 - 5.42). However, polyribosomes (Figs. 5.38, 5.40 and 5.41), nuclei, nuclear envelopes and, especially, nuclear pores remained reasonably intact at all concentrations of T-X (Figs. 5.36a, b and 5.39). The latter were often associated with microtubules (Fig. 5.36b), as was observed in non-detergent-treated tissue (Fig. 5.35) and in conventional preparations (see Sections 2.3.5 and 2.3.6). T-X also had no deleterious effect on cortical microtubules; indeed, with increasing concentrations of the detergent, they became easier to visualise, owing to the degradation of the surrounding cytoplasm (Figs. 5.38, 5.40 and 5.41). Furthermore, despite the slight
plasmolysis, in the 0.2 and 1% T-X treatments, the cortical microtubules still appeared to be congruent with nearby microfibrils (e.g., Fig. 5.40). In both the 5% T-X and the 1% T-X 'pre-treated' samples (which were similar in appearance to each other), recently deposited wall material had dispersed slightly (Fig. 5.42), but this did not occur in the other treatments.

Paraformaldehyde in MSB plus 0.5% T-X was thus chosen for use in the immunofluorescence experiments, as it seemed that it would give a reasonable level of preservation whilst also permeabilising the cytoplasm sufficiently to allow antibody penetration. Indeed, fibrillar staining with anti-tubulin antibodies (see below) was observed when such a fixative solution was used. When T-X was omitted no staining occurred. However, when MSB was used in the fixative, samples had to be stored in EGTA overnight to aid squashing.

Autofluorescence of the opened-out cells in these experiments, as in the hand sections (Section 5.3.1), was generally very low (Figs. 5.43a, b) and not structured, even in the occasional slightly brighter sample (Figs. 5.44a, b). Usually this was also the case after the addition of RAM-FITC (Figs. 5.45a, b) except in some incompletely dissected cells where the fluorochrome may not have been properly rinsed out, but, even in these cells, no fibrillar fluorescence was apparent (Figs. 5.46a, b). In addition, brightly fluorescent contaminating particles were sometimes found either adherent to the PLL or seemingly trapped between the PLL and folds in the opened-out walls. These patterns were not affected by either the different fixatives employed or the BSA pre-treatment.

With the anti-tubulin MABs, YOL 1/34, YL 1/2 (the Kilmartin MABs) and TU-01, slight cytoplasmic, strong nuclear and bright fibrillar fluorescence was observed, although the form of the fibrillar fluorescence did vary. The Kilmartin MABs both gave a fine pattern (e.g., Fig. 5.53a). By
comparison, TU-01 was brighter and coarser, perhaps because it was revealing larger groups of microtubules, but equally its apparent superiority could be because the anti-mouse conjugate bound more effectively to it (the Kilmarlin MABs were raised in rat, whose IgG has only a 50% cross-homology with the conjugate used [Dr. S. Wick, R.S.B.S., A.N.U., pers. comm.]). Fibrillar staining was not merely a property of cytoplasmic staining in general (which was low anyway) as shown by the differences in the pattern of phase and fluorescence images (e.g., Figs. 5.50d, f). Nor was it a wall-related phenomenon, as displaced cytoplasm frequently showed fibrillar patterns (Figs. 5.47a, b). This also demonstrates that care must be taken to ensure that cytoplasm showing fibrillar staining is still in its original position in vivo against the appropriate wall.

Absorption of the TU-01 MAB with both levels of mung bean tubulin eliminated fibrillar fluorescence, but low-level binding to the cytoplasm and bright nuclei was still apparent (Figs. 5.48a, b). Fluorescence with any of the MABs was not significantly affected by either brief air-drying of the samples after the cutting-open of the cells or the use of a BSA pre-treatment and so they were not generally carried out. The MAB K207B8 did not give fibrillar staining. This MAB has been found to work well, using the method of Wick et al. (1981), on some, but not all, higher plant species, (Dr. K. Mizuno, pers. comm.).

It was thus concluded from the electron microscopic and pre-absorption studies that the fibrous fluorescence provided a satisfactory indicator of microtubular arrays. Cells from unwounded and day 0 stems exhibited areas of approximately transverse fibrillar staining against their side walls, presumably reflecting the position of cortical microtubules. Two such examples, each encompassing around 9,000µm² of wall, are shown in Figures 5.49a and 5.50d. Whilst the former is strictly transverse, the latter is
at a slight angle, but this may be the consequence of minor displacement of
the cytoplasm (Fig. 5.50f). Polarisation (Figs. 5.49c and 5.50a) and
interference examination of these and other samples showed that such cells
always exhibited the typical day 0 side wall characteristics of transverse
reinforcement with oval pits and (externally positioned) longitudinal
thickenings (see Section 3.3.1). In no samples were groups of
longitudinally-oriented or steeply-angled microtubules observed. The
images thus support the conclusion reached from conventional electron
microscopic preparations, that cortical microtubules are transversely
oriented or else arranged in a shallow helix. They provide no indication
of a helicoidal arrangement of microtubules (gradual changes in
orientation) as fluorescent fibrils at all points of the compass would be
expected in different samples. The visualisation of staining pattern in
end walls was inhibited by technical difficulties in both pre- and post-
wounding samples.

After wounding, a major change in the orientation of cortical
microtubules occurred, as revealed by the pattern of fibrillar
fluorescence. Many cells, such as the day 2 example in Figure 5.54d, had
areas of longitudinal fibres running down a portion of the side wall.
Polarisation (Fig. 5.54a) and interference optics (Figs. 5.54b, c) reveal
that the cell has exhibited the typical wound response (as described in
Section 4.3.1) with longitudinal cellulose deposition reducing retardation,
increasing optical thickness and altering pit shape in the side walls, this
occurring to a greater extent in two portions of the wall. The area of
fluorescence is in a zone of lesser change and is internal to a broad
external thickening (Figs. 5.54b, c). Also observed were cells with groups
of transverse and longitudinal microtubules at different locations around
the perimeter of the side wall, such as the example shown in Figures 5.53a,
b. Polarisation (Fig. 5.53d) and interference microscopy (Figs. 5.53e, f)
confirm that the usual wound response had occurred in this cell.

The coarser, but brighter, staining pattern of TU-01 compared with the Kilmartin MABs of the other images, is shown in the day 2 cell in Figure 5.51a where areas of transverse microtubules, as well as others radiating out from the (brightly fluorescent) nucleus, can be seen. Another, day 3, TU-01 example has areas of both transverse - by the nucleus (Fig. 5.52a) - and longitudinal (Fig. 5.52b) microtubules, although the latter may have been partially disturbed by the displacement of adjacent cytoplasm (Fig. 5.52c). This again demonstrates the presence of both longitudinal and transverse microtubule populations at the same time, but in different locations in a single responding cell.
5.4 DISCUSSION

With any immunocytochemical study two important questions should be posed, especially where, as here, they involve novel techniques. First, is antibody binding specific to the cellular component that it is being directed against and second, does tissue preservation adequately reflect the condition *in vivo* of the cells?

It is apparent from observations of control treatments that fixed *D. stramonium* pith cells are not significantly autofluorescent and do not bind fluorochrome or gold or the associated rabbit IgG to any great extent. The use of hapten sugars shows that the lectin that has been isolated from seeds of *D. stramonium* (Kilpatrick and Yeoman, 1978), if present in pith cells, does not bind Ig. That the cells do not possess generalised protein binding sites is shown by the null or minimal effect of the BSA treatments, CC4 being somewhat exceptional. As CC4 is the only antibody that binds non-specifically, it seems likely to be a characteristic of the antibody rather than of the cytoplasm *per se*.

Mouse (and rat) Ig binds strongly to the nuclei and slightly to the cytoplasm of *D. stramonium* pith cells. This is exemplified by the diffuse binding of the irrelevant MAB ACR, but is also illustrated by the continued non-specific binding of CC4 and TU-01 after elimination of their specific fibrillar staining by pre-absorption with muscle F-actin or plant tubulin respectively. These experiments also provide strong evidence that the recognition site of each antibody is binding to an epitope on the relevant cytoskeletal protein, rather than on an associated molecule and that the non-specific binding is a property of another part of the Ig molecule in the case of TU-01 and mainly so, but not completely, with CC4. In the case of the anti-tubulin MABs, YL 1/2, YOL 1/34 and TU-01, the existence of this specificity is further supported by their specific binding in blotting...
experiments to the $\alpha$-subunits of plant tubulin (homologous, but not identical to the $\alpha$-subunit of mammalian brain tubulin) (Mizuno et al., 1985a).

It is well documented that the cyclic peptide phallotoxins and their fluorescent derivatives such as NBD-Ph bind specifically to F-actin, at up to equimolar ratios (Barak et al., 1980; Wieland and Faulstich, 1978). The fibrillar staining observed in the present work with NBD-Ph, and the lack of such patterns in controls, is further evidence that the CC MABs are reacting with F-actin in $D. stramonium$ pith cells. As was the case with the CC antibodies, not all NBD-Ph-treated cells showed staining. It seems most unlikely that some cells would possess actin filaments while others did not. Variation could be caused by differences in antibody penetration. NBD-Ph similarly shows great variability in its staining, even in cells that are as apparently uniformly accessible as those in $T. trandescantia$ stamen hairs (Prof. B.E.S. Gunning, R.S.B.S., A.N.U., pers. comm.). The quality or actin preservation may also vary from cell to cell.

It is notable that CC4 stained the site of dictyosomes whilst NBD-Ph appeared not to do so. In this case it is possible that the antibody may be binding an epitope in a protein other than actin. MABs are known to be subject to this phenomenon (e.g., Lane and Koprowski, 1982; Nigg et al., 1982). However, Bendayan (1983) has shown that anti-actin antibodies label the Golgi apparatus and associated vesicles in pancreatic endocrine and exocrine cells. The lack of punctate staining with NBD-Ph does not necessarily mean that F-actin in absent at the dictyosomal sites: it may be that the amount is so low that it is detectable only with the amplification that is provided by the immunocytological methods (cf. Barak et al., 1981).

Having determined, to a reasonable degree of satisfaction, that the antibodies directed against the cytoskeletal proteins are binding
specifically, the question of cellular preservation arises. In the case of
the hand-sectioning experiments, a degree of cytoplasmic distortion was
inevitable in cut cells, but many of them still exhibited features typical
of wound response (cf. Chapter 2). Systrophes never survived in cut cells,
but fortunately could be studied in uncut cells in the NBD-Ph
experiments. The protocol used for applying anti-tubulin to opened-out
cells may initially seen harsh, but other current procedures for
immunofluorescent localisation of tubulin in plant cells are also quite
severe, employing EGTA or enzyme incubations, squashing and air-drying
(e.g., Clayton and Lloyd, 1984, 1985; Gunning and Wick, 1985; Lloyd, 1983;
Wick, 1985a, b; Wick and Duniec, 1983, 1984; Wick et al., 1981, 1985). The
possibility of cytoplasmic disturbance during processing has not been
explicitly considered in many such studies, although it has been alluded to
(e.g., see the legend for Fig. 1d of Lloyd, 1983). The tendency for
cytoplasmic displacement in the current study increased with the use of T-X
in the fixative, but, as demonstrated by EM, this was required to obtain
sufficient cytoplasmic permeability to allow antibody penetration.
Moreover, the electron microscopic studies also showed that cortical
microtubules survived the T-X treatment.

Having established that the antibodies used do indeed reveal the
distribution of microtubules and F-actin within the cells, the role of
these components in the wound response may be discussed.

It is apparent that F-actin is associated with clustered nuclei both
before and after traumatotaxis. Actin-containing strands could be involved
in chloroplast movement, as may be inferred from scanning electron
microscopic images (Fig. 2.27b), or nuclear migration, or both. In other
systems drug studies point to traumatotaxis being an actin-based phenomenon
(e.g. Schnepf and von Traitteur, 1973). This is also the case for the
formation of systrophes in some species (Weidinger, 1980a). In the current study, F-actin was observed in the cytoplasmic strands that preceded and, later, formed stars. This could imply that an actin-based motility system participates in positioning the nucleus in the centre of the cell prior to division. In this regard, cytoplasmic streaming, which is presumed to be actin-based (Williamson, 1980), was observed in these strands when living cells were viewed in thick hand-sections (Section 2.3.5). However, it may be that the nucleus is the fulcrum of cytoplasmic streaming and that the actin filaments maintain links with the nucleus whatever its position - with some other mechanism being responsible for nuclear movement (cf. Section 2.4). Also of interest was the staining of a phragmoplast with CC2. Evidence is accumulating (Clayton and Lloyd, 1985; Gunning and Wick, 1985) that F-actin is involved in cell plate formation. These main findings from the use of the actin tracers on hand sections represent information which would have been difficult to obtain with other techniques such as EM.

In cultured animal cell monolayers, the Golgi apparatus and associated MTOC lie in front of the nucleus during cell response to wounding (Gotlieb et al., 1981, 1983; Kupfer et al., 1982). Although dictyosomes, as visualised by CC4, were clustered around nuclei, as well as scattered through the cytoplasm, there was no indication that they are a vanguard of nuclear migration.

A similar distribution was observed using EM: binding of gold to dictyosomes occurred with CC4 in the experiments on pre-embedding labelling. The other binding observed in such experiments was probably non-specific in the main. No putative F-actin was seen and so it may well not have survived processing. This also seemed to be the case in the experiments on post-embedding labelling of C. corallina, using both SM at
the light microscopic level (present study) and Spurr's or Lowicryl K_4M embedded material at the electron microscopic level (Craig, 1984). That chemical changes occur in the tissue during processing, which could render the actin unrecognisable to the antibodies, is illustrated by the alteration of the wavelength of the chlorophyll autofluorescence in the SM-embedded material. Similar problems of antibody recognition appear to be a general phenomenon in experiments using post-embedding techniques for the localisation of actin, as there are no reports of such labelling having been successful with plants and only a few with animal tissue (Bendayan, 1983, 1984; Wolosewick and De Mey, 1982; Wolosewick et al., 1983).

Despite a degree of cytoplasmic disturbance and some variable staining, images obtained by applying anti-tubulin to opened-out cells reveal the dramatic change in the pattern of the cortical microtubules that occurs during the wound response. Prior to wounding, microtubules were seen to be transverse against the longitudinal walls, mirroring the overall cellulose orientation. After wounding, the cells laid down a polylamellate wall with both longitudinal and transverse layers of microfibrils (Section 4.3.3). Microtubules distributed in similar fashion were revealed in fluorescence images of cells that were shown by subsequent examination of their wall characteristics, using polarisation and interference optics, to be responding to the wound. This is a novel feature of the present experiments in that published techniques for immunocytochemical localisation of plant microtubules involve cell wall degradation.

The ability to visualise both cellulosic and microtubular changes within individual cells is potentially valuable in the study of plant morphogenesis. Congruency of such alterations has been observed, using polarised light and EM, to determine microfibril and cortical microtubule orientations respectively during an induced polarity shift in epidermal
cells undergoing organogenesis (Hardham et al., 1980). A similar situation occurs in the present system. The data point to an inextricable link between cortical microtubules and the spatial organisation of wall deposition. It has been suggested (e.g., Emons and Walters-Arts, 1983; Neville and Levy, 1984) that no such link exists in the case of polylamellate walls, which are held to be laid down in a helicoidal manner either with (Lloyd, 1984a) or without (Neville and Levy, 1984; Roland and Vian, 1979) microtubule guidance (see Chapter 4). In the current system EM has shown the walls to be polylamellate, but composed of discrete layers of similarly-aligned microfibrils with major shifts between successive lamellae (Section 4.3.3). Furthermore, adjacent cell faces within individual cells deposit cellulose in different directions at the same time, this being indicated by the distribution of the cortical microtubules (Section 4.3.5). The immunofluorescence images confirm the occurrence of populations of microtubules aligned at 90° to each other on different wall faces. It seems inescapable that the possibility of a helical or helicoidal template controlling post-wounding wall deposition in *D. stramonium* pith cells can be excluded.
FIGURE LEGENDS - CHAPTER FIVE

All abbreviations and conventions for the figures presented are as used in previous chapters.
Figures 5.1 - 5.9:

Localisation of actin in transverse hand-sections: controls.

All figures are from day 3 wounds; (a) fluorescence and (b) phase images. All pictures are oriented as if the wound was up and down the left-hand side of the page. Note that intact systrophes are only present in cells that have not been cut open upon sectioning.

Figure 5.1:

Autofluorescence: timed exposure showing the very low level of intrinsic fluorescence. One cell contains a systrophe (arrow), the other a recent division (arrowhead). x 300.

Figure 5.2:

Autofluorescence: automatic exposure revealing the diffuse, structureless nature of the cytoplasmic autofluorescence and the brighter nuclei (arrows). The middle nucleus is in a systrophe, the two adjacent ones are traumatotactic. x 300.

Figure 5.3:

Autofluorescence: automatic exposure at a lower magnification than Figure 5.2 showing the variability of nuclear fluorescence levels (e.g., arrows). x 120.

Figure 5.4:

Conjugate (RAM-FITC) only: automatic exposure. There is little change in the form of the fluorescence from Figure 5.3, apart from the introduction of the occasional contaminating particle (arrowhead). Nuclear fluorescence (e.g., arrows) is still variable. x 120.

Figure 5.5:

Conjugate (RAM-FITC) only: timed exposure showing that the very low level of autofluorescence is little altered by the addition of the conjugate. x 90.

Figure 5.6:

ACR: automatic exposure. The distribution of fluorescence is non-specific and unrelated to that shown by the antibodies directed against the cytoskeleton (e.g., Figs. 5.10 - 5.12). Arrows, nuclei; arrowheads, contamination from the RAM-FITC. x 120.

Figure 5.7:

ACR plus hapten sugars: automatic exposure. The non-specific fluorescence seen in Figure 5.6 remains. x 230.

Figure 5.8:

ACR: timed exposure. The intensity of the fluorescence is low compared with the antibodies directed against the cytoskeleton (e.g., Figs. 5.10 - 5.12). x 120.

Figure 5.9:

ACR plus 4 x F-actin: automatic exposure: The non-specific fluorescence seen in Figure 5.6 remains. x 120.
Figures 5.10 - 5.14:

Localisation of actin in transverse hand-sections: CC MABs at low magnification.

All figures are from day 3 wounds; (a) fluorescence and (b) phase images. All pictures are oriented as if the wound was up and down the left-hand side of the page.

Figure 5.10:

CC2: automatic exposure. Nuclear and cytoplasmic fluorescence have increased, but some fibrillar fluorescence is also present (e.g., arrows). The bright walls under the wound (arrowhead) exhibit the autofluorescent characteristics of suberin. x 160.

Figure 5.11:

CC4: automatic exposure. Nuclear and cytoplasmic fluorescence have increased, but most apparent is bright, punctate fluorescence that presumably represents the site of dictyosomes (see Craig, 1984). x 160.

Figure 5.12:

CC4 + BSA: automatic exposure. Much of the background cytoplasmic-fluorescence apparent in Figure 5.11 has been reduced by the BSA pre-treatment. Cells not showing the bright staining pattern (e.g., star) are those wholly within the thickness of the section. x 160.

Figures 5.13 and 5.14:

CC4 + 4 and 40 x F-actin: timed exposures. In both cases, pre-absorption of the antibody with F-actin virtually obliterates cytoplasmic staining, although variable nuclear fluorescence remains (e.g., arrow). All x 120.
Figures 5.15 - 5.18:

Localisation of actin in transverse hand-sections: CC MAbS at high magnification.

All figures are from day 3 wounds; (a) fluorescence and (b) phase images. All pictures are oriented as if the wound was across the top left-hand corner of the page.

Figure 5.15:

CC4: automatic exposure. Collapsed systrophe in a cut cell. Dictyosomes (arrowhead) and fluorescent fibres (arrow) can be seen in the vicinity of the nucleus (N). (Changing the plane of focus, whilst actually viewing the specimen, allowed the identification of the collapsed star as such, rather than as, say, an organelle-enclustered nucleus by an end wall). x 465.

Figure 5.16:

CC4 + BSA: automatic exposure. Nucleus (N) positioned in a phragmosome. Many dictyosomes are present (e.g., arrowhead) especially around the nucleus. That the pattern of fluorescence observed is not simply because of staining of the cytoplasm per se is shown by the non-visibility in (a) of the arrowed strand in (b). x 465.

Figure 5.17:

CC2: automatic exposure. A phragmoplast (arrowhead) is stained. The pattern of fluorescence shows that the two daughter nuclei are not responsible for the image. Other cytoplasm within the cell (e.g., arrow) is not stained. x 465.

Figure 5.18:

CC2: automatic exposure. Nuclei (N) undergoing systrophic formation after the first round of division (new cross-walls marked by arrowheads). Fluorescent fibres are seen (e.g., arrows) in the cytoplasmic strands associated with the nuclei. x 465.
Figures 5.19 - 5.24:

Localisation of actin in transverse hand-sections: video microscopy and NBD-Ph.

All figures are from day 3 wounds; (a) fluorescence and (b) phase images. All pictures are oriented as if the wound was across the top of the page, unless otherwise stated.

Figure 5.19:

CC2: organelle-enclustered nucleus (N) by an end wall (E) with an associated fluorescent fibre (arrow). x 700.

Figure 5.20:

Autofluorescent control; NBD-Ph experiment. Despite video-enhancement of the signal, the autofluorescence characteristics remain as in the antibody experiments (see Figs. 5.1 - 5.3) with very low structureless cytoplasmic- and some nuclear-fluorescence. The nucleus (N) is in a systrophe in an uncut cell. x 300.

Figure 5.21:

NBD-Ph + Ph control. Fluorescence is structureless and of a fairly low intensity, although the nucleus (N; in a systrophe) is somewhat brighter than the cytoplasm. x 700.

Figure 5.22:

NBD-Ph. A nucleus (N) is a thickened phragmosome containing a much finer fluorescent strand (arrows). This image is oriented as if the wound was up and down the left-hand side of the page. x 700.

Figure 5.23:

NBD-Ph. An early- or else an asymmetrically-positioned- systrophe. A relatively thick cytoplasmic strand (arrow in (b)) containing a fine fluorescent strand (arrow in (a)) can be seen traversing the vacuole to the nucleus (N). x 700.

Figure 5.24:

NBD-Ph. A systrophe with fluorescent fibres (arrows) in its radiating cytoplasmic-arms. x 700.
Figures 5.25 - 5.28:

Localisation of actin in transverse hand sections: pre-embedding labelling for EM using an immunogold procedure.

All figures are from days 2 or 3 wounds.

Figure 5.25:

'Gold-only' control, showing the only label (arrow) found in 30 cells examined in detail, which had been identified as having been cut open. x 40,000.

Figure 5.26:

CC4-gold. A labelled (e.g., arrows) nucleus, although the nucleolus only possesses label at its periphery. The nuclear envelope is ruptured in places (e.g., arrowhead). x 15,000.

Figure 5.27:

CC4-gold. Label (e.g., arrows) at the periphery of an amyloplast (identified as such by its characteristic appearance following conventional staining for EM). x 45,000.

Figure 5.28:

CC4-gold. Label (e.g., arrows) is present on vesicles which are near a, rather poorly-preserved, dictysome (star). The ruptured plasma membrane is also labelled (e.g., arrowhead). x 60,000.
Figures 5.29 - 5.33:

Localisation of actin in transverse hand-sections: pre-embedding labelling for EM using an immunogold procedure.

All figures are from days 2 or 3 wounds.

Figure 5.29:

CC4-gold. Labelling (e.g., arrow) of vesicles is apparent. x 60,000.

Figure 5.30:

CC4-gold. Label (e.g., arrows) on a plasmolysed, but still relatively intact, plasma membrane. x 30,000.

Figure 5.31:

CC4-gold. Microtubules (e.g., arrowhead) survive the protocol and are not labelled. x 40,000.

Figure 5.32:

CC4-gold. Structures bearing an immediate resemblance to F-actin do not appear to survive processing. However, filaments of indeterminate nature, which could represent poorly-preserved F-actin, are labelled (e.g., arrow). x 85,000.

Figure 5.33:

CC4-gold. As Figure 5.32; labelled vesicles (e.g., arrowheads) can also be seen. x 30,000.
Figures 5.34 - 5.36:

EM of cells preserved in fixative with or without T-X.

All figures are from transverse sections of days 2 or 3 wounds.

Figure 5.34:

No T-X. Nucleus (N) by a recent, periclinal, cross-wall (asterisk). Preservation is of a reasonable standard with intact nuclear- (arrow) and organelle- (arrowheads) membranes. A, amyloplast; D, dictyosome; M, mitochondrion. x 20,000.

Figure 5.35:

No T-X. Grazing view of a nucleus (N) showing many nuclear pores (e.g., arrows) and microtubules between the nucleus and the cell wall (e.g., arrowhead). x 30,000.

Figure 5.36:

Plus 0.2% T-X.

(a) A similar view to that in Figure 5.35. A nucleus (N) is seen in grazing section with many nuclear pores (e.g., arrow) and associated microtubules (arrowhead, see also (b)). Although the appearance of the nucleus is little changed by the addition of 0.2% T-X, the amyloplasts (A); dictyosomes (e.g., D) and mitochondria (e.g., M) are slightly degraded. x 9,000.

(b) Higher magnification showing microtubules extending from the nuclear surface into the cytoplasm (arrowheads). x 25,000.
Figures 5.37 - 5.42:

EM of cells preserved in fixative with T-X.

Figure 5.37:

Plus 0.2% T-X. Slight plasmolysis (star) and disruption of the plasma membrane (arrow) and amyloplast (A) membranes (e.g., arrowhead) are apparent. Some extraction of the cytoplasmic ground substance has also taken place. x 22,000.

Figure 5.38:

Plus 0.2% T-X. Microtubules (e.g., arrowhead) are well preserved as are polyribosomes (e.g., arrow). On the other hand, mitochondria (e.g., M) and the cytoplasmic ground substance are slightly degraded. x 22,000.

Figure 5.39:

Plus 1% T-X. The nucleus (N) is reasonably preserved, but most of the cytoplasmic organelles are not. Dictyosomes (e.g., asterisk) and mitochondria (e.g., arrowhead) are recognisable, but the site of chloroplasts can only be determined by their remnant starch grains (e.g., arrow). x 7,000.

Figure 5.40:

Plus 1% T-X. Microtubules (e.g., arrowhead) are well preserved and, despite some plasmolysis (star), remain parallel to nearby microfibrils (arrow). x 32,000.

Figure 5.41:

Plus 5% T-X. Most cytoplasmic organelles are severely degraded, although mitochondria (e.g., M) are still just recognisable. However, microtubules (e.g., arrowhead) and polyribosomes (e.g., arrow) are well preserved. x 22,000.

Figure 5.42:

Plus 5% T-X. The severe extraction of the cytoplasm and considerable plasmolysis (star) can be seen. No plasma membrane is visible. Recently deposited wall material, both in the new cross-wall (asterisk) and in the thickened mother cell wall (R), has dispersed (e.g., arrows) with the addition of 5% T-X. x 14,000.
Figures 5.43 - 5.49:

Localisation of tubulin in opened-out cells.

Figures 5.43:

(a) Day 0; autofluorescence: timed exposure showing the very low level of intrinsic fluorescence that is found in most cells.
(b) Köhler image of (a). (a) and (b) x 300.

Figure 5.44:

(a) Day 2; autofluorescence: automatic exposure revealing the diffuse structureless nature of the cytoplasmic autofluorescence even in slightly brighter samples.
(b) Phase image of (a). (a) and (b) x 300.

Figure 5.45:

(a) Day 0; conjugate (RAM-FITC) only: timed exposure. The level of autofluorescence is little altered by the addition of conjugate.
(b) Phase image of (a). (a) and (b) x 300.

Figure 5.46:

(a) Day 2; conjugate (RAM-FITC) only: automatic exposure. This cell has been incompletely dissected - only one end wall (asterisk) has been removed - and fluorochrome (e.g., arrowhead) remains trapped within the cell. Nevertheless, the fluorescence is still non-fibrillar and not structured.
(b) Phase image of (a). (a) and (b) x 300.

Figure 5.47:

(a) Day 0; YL 1/2: automatic exposure. The cytoplasm, which has been displaced from the wall (star), exhibits fibrillar fluorescence (arrow). This demonstrates that the antibody-induced fluorescence is associated with the cytoplasm and not the wall.
(b) Köhler image of (a). (a) and (b) x 465.

Figure 5.48:

(a) Day 2; TU-01 plus 50 x tubulin: automatic exposure. Fibrillar fluorescence is eliminated by the pre-absorption of the antibody with plant tubulin, but bright nuclear- (N) and some cytoplasmic- (arrow) fluorescence remains. The cell has been incompletely dissected and some trapped fluorochrome (arrowhead) is also present.
(b) Phase image of (a). (a) and (b) x 300.

Figure 5.49:

(a) Day 0; YL 1/2: automatic exposure. A group of transversely-oriented microtubules (e.g., arrow) are seen traversing part of the side wall.
(b) Phase image of (a).
(c) View of the opened-out cell from which (a) and (b) are taken (marked area) as seen in polarised light. The cell has the typical negative retardation and transversely-elongated pits (e.g., arrow) of unwounded samples (see Chapter 3). The refractile granules (e.g., arrowhead) are probably crystals, originating from the PBS, ensnared in the Mowiol mounting medium. (a) and (b) x 830; (c) x 220.
Figures 5.50 - 5.52:

Localisation of tubulin in opened-out cells.

All fluorescence images are from 'automatic' exposures.

Figure 5.50:

Day 0; opened-out cell consisting of a complete side wall and one end wall (E).

(a) As seen in polarised light, the area containing the microtubular fluorescence shown in (d) is marked. The side wall shows the transverse reinforcement, oval pits (e.g., white arrow) and longitudinal bands (e.g., black arrows) characteristic of unwounded samples (see Chapter 3).

(b) As seen in uniform field interference. The longitudinal bands in the side wall are even more apparent than in (a).

(c) In fringe field interference, the longitudinal bands (examples of positions marked by arrows) can be seen to be slightly thicker than the adjacent wall faces.

In summary (a) - (c) show that this cell is typical as regards its wall characteristics. Note that, although the smaller portion of side wall and the end wall are at the same location, the main piece of side wall was rotated through 180° during transfer for examination in polarised light and interference optics. Thus, the area of wall possessing the fibrillar fluorescence is upside down in (d) - (f) relative to (a) - (c). This is shown by the star and asterisk marking the same points in (a) as in (f).

(d) A portion of the side-wall (marked in (a)) after staining with YL 1/2. A group of transversely- or nearly-transversely-, oriented microtubules (e.g., arrow) can be seen.

(e) Köhler image of (d).

(f) Lower magnification phase-image of (d) and (e). A fold in the wall (arrowhead) may have slightly displaced the cytoplasm containing the group of stained microtubules seen in (d). (a) - (c) x 130; (d) and (e) x 830; (f) x 165.

Figure 5.51:

(a) Day 2; TU-01; the brighter, coarser-staining pattern of this antibody compared with YL 1/2 (e.g., Fig. 5.50d) and YOL 1/34 (e.g., Fig. 5.53a) is apparent. Microtubules are seen in a transverse orientation (e.g., arrows) and also radiating out (e.g., arrowhead) from the brightly fluorescent nucleus (N).

(b) Phase image of (a). (a) and (b) x 435.

Figure 5.52:

(a) Day 3; TU-01. Longitudinally-oriented microtubules can be seen (e.g., arrow). Nearby cytoplasm (asterisk) has been pulled off the wall, but the fibrillar area appears to have remained largely in situ.

(b) The same wall, but a different area from that seen in (a). Transversely-oriented microtubules are present (e.g., arrow) by a brightly, fluorescent, nucleus (N). (a) and (b) x 435; (c) x 315.

(c) Phase image of the side wall from which (a) and (b) are taken. The nucleus (N) present in (b) and the displaced cytoplasm asterisked in (a) can be seen.
Localisation of tubulin in an opened-out cell.

Day 2; YOL 1/34. In order to facilitate recognition of the area of fluorescent staining, the star marks the same position in all images.

(a) and (b). Fluorescence images at two different levels of focus showing transversely- (e.g., arrows) and longitudinally - (e.g., arrowheads) oriented microtubules. A brightly fluorescent nucleus (N) is also present.

(c) Köhler image of the area encompassed by (a) and (b).

(d) The same wall as seen in polarised light. A typical day 2 wound response (see Chapter 4) is apparent with reduced retardation and a change in pit shape (e.g., arrow).

(e) In fringe field interference, the longitudinal bands seen in (f) (examples of positions marked by arrows) are thicker than the adjacent wall faces. Overall fringe deflection is greater than in unwounded samples (e.g., Fig. 5.50c) owing to the post-wounding deposition that has taken place.

(f) In uniform field interference, longitudinal bands, which, owing to the post-wounding deposition, are barely visible in (d), are apparent (e.g. arrows). (a) and (b) x 650; (c) x 500; (d) - (f) x 300.
Figure 5.54:

Localisation of tubulin in an opened-out day 2 cell.

(a) As viewed in polarised light. The area of wall from which (d) and (e) are taken is marked. The cell exhibits the typical wound-response with reduced retardation, a change in pit shape (e.g., arrow) and greater change in two areas of wall (asterisks).

(b) In uniform field interference, longitudinal bands can be seen (e.g., arrows), one of which (star) underlies the portion of wall shown in (d) and (e).

(c) In fringe field interference, the longitudinal bands (examples of positions marked by arrows) are thicker than the adjacent wall faces.

In summary, (a) - (c) show that the cell was undergoing post-wounding deposition in the usual manner.

(d) A portion of the side wall (marked in (a)) after staining with YOL 1/34. Longitudinally-oriented microtubules are present (e.g., arrows) running down the length of the wall.

(e) Kohler image of (d). (a) - (c) x 250; (d) and (e) x 680.
CHAPTER SIX

CONCLUDING COMMENTS
The response to tissue wounding of stem pith cells of *D. stramonium*, as elucidated in this study, may be summarised as follows.

Prior to wounding, the cells of the recently-matured, i.e., just having ceased elongating, internode are relatively large, averaging around 250 µm in length and 100 µm in diameter. In a detailed study of cell diameter, an average diameter of 135 µm was obtained for (day 1) cells of the internode above the crotch (cf. below the crotch in the present report) of *D. stramonium* (Warren Wilson and Grange, 1984). The cells are highly vacuolated with only a thin layer of cortical cytoplasm, most prominent in which are the single, flattened elipsoidal nucleus lying against any wall of the cell, and several amyloplasts mainly found near the basal end wall. The fact that the nucleus does not similarly sink to the bottom of the cell may be indirect evidence of a nuclear anchoring system as envisaged by Lloyd and Barlow (1982).

The longitudinal walls of the cell are transversely-reinforced overall with cellulose, with sparse, but congruently-aligned, cortical microtubules. At the margins of the intercellular spaces, which occur at the vast majority of cell corners, external ribs of longitudinal cellulose deposition are present. As the outer layer, such microfibrils must have been laid down early in the development of the cell. Indeed, in coleoptiles of *Avena*, thickenings have been observed in isodiametric meristematic cells (Mühlethaler, 1950). This means that passive reorientation, as a component of the multi-net growth hypothesis of Roelofsen and Houwink (1953), cannot be solely responsible for the formation of the thickenings as insufficient extension would have occurred in such young cells. However, in order for the ribs to have remained prominent during extension growth they must have been supplemented by microfibrils other than those initially deposited in a longitudinal fashion.
at cell corners. Passive reorientation alone cannot of itself explain the maintenance of the ribs. They appear to form a buttress with no gradual change of microfibril orientation at their edges. Drawing or squeezing of earlier-deposited microfibrils into the cell corners may occur, as envisaged by Wardrop and Cronshaw (1958) and Roelofsen (1958). Such a mechanism when combined with passive reorientation of older microfibrils may explain the persistence of the ribs. The longitudinal walls are also specialised internally at the site of intercellular spaces, possessing greater amounts of cellulose (see also Deshpande, 1976b) and, probably, matrix substances than the adjacent wall faces. Transverse lamellae of microfibrils are interspersed with layers mainly composed of matrix substances. This can give a false appearance of (microfibrillar) polylamellation.

Transversely-elongated, lens-shaped pits are found along cell faces and at the margins of intercellular spaces, although not at the spaces themselves. The pattern of cellulose deposition at the side-wall pits is consistent with a minimal disruption of transverse reinforcement. If membrane-flow is responsible for controlling the orientation of deposition, as proposed by Mueller and Brown (1982a, b), it must be bi-directional rather than uni-directional in order to account for the images of pits obtained from replicas of opened-out cells.

By contrast, transverse (end) walls have no overall cellulosic order, showing only local grouping of (straight) microfibrils and co-aligned cortical microtubules. Small, roughly-circular pits are found scattered across the walls. Oblique end walls, which, although in a minority compared with transverse walls, are fairly common at the ends of cell packages, are intermediate between transverse and longitudinal walls in character. They possess some degree of overall order and their pits,
slightly larger than those in transverse walls, are arranged approximately into rows. The characteristics of oblique end walls are consistent with their being influenced by a spillover of transversely-, or nearly transversely-, aligned cortical hoops of microtubules and nascent microfibrils from the adjacent side walls. The integrity of hoops and 'spillover' of such structures from one category of wall face to another is of importance in the wound response of the cell.

After tissue wounding in the *D. stramonium* pith cells, events occur in the following chronological sequence. Within a few hours of wounding, clustering of the amyloplasts around the nucleus takes place. This may be an actin-based phenomenon. The organelle-enclustered nuclei then migrate around the side walls of the cell until, by day 1, the vast majority of cells, lying in a zone extending from immediately below the cut surface to several files back from it, exhibit this traumatotaxis. This migration may also be actin-based and, in SEM images, traumatotactic nuclei appear to be encased in a cage which may contain cytoskeletal components. During these initial enclustering and migration phases, considerable swelling and rounding of the nucleus occur, together with an increase in nuclear pore density. (As nuclear surface area also appears to rise, the total number of nuclear pores must increase substantially.) Traumatotaxis does not occur in response to transverse wounds, where there is no subsequent polarity change, so this movement, at least, is a manifestation of the change in polarity.

The reason for the organelles clustering around the nucleus is unclear. Teleologically, it could contribute to an equitable partition of organelles upon division. However, mitosis occurs up to 3 or 4 days later. Alternatively, focussing the cell's carbohydrate reserves around the nucleus could contribute to the increase in metabolism that has been
observed in other wound systems (e.g., Lipetz, 1970). However, an alternative possibility is that the increased mass of the associated organelles in some way aids in stress-detection when the nucleus is suspended in a systrophe and the division site is being established. In this regard, clustering occurs in *Nautilocalyx* epidermal explants after the nucleus has come to reside in the centre of the cell, but the organelles disperse before the onset of mitosis (Venverloo et al., 1980). This suggests a transient function for the enclustered nucleus, which would correlate with the proposed detection of stress during the systrophic stage (Chapter 2).

After clustering and traumatotaxis have occurred, thin, F-actin-containing, cytoplasmic strands form and traverse the vacuole to focus on the nucleus. This, with its associated organelles, then comes to reside in the spatial centre of the cell in a star-like structure—a systrophe—of narrow, radiating, cytoplasmic strands. These contain microtubules and F-actin and exhibit cytoplasmic streaming. Some of the arms of the systrophe predict the line of the future cell plate and may possibly play a significant role in determining its position, via the division site. They, or the cytoskeletal components within them, could detect or transmit stresses. The strands apparently coalesce to form a raft of cytoplasm across the vacuole in which cytokinesis subsequently takes place. A PPB of cortical microtubules forms prior to strand fusion and remains in position at the perimeter of the phragmosome until early spindle formation. Microtubules are seen around the periphery of the nuclear envelope from traumatotaxis until pre-prophase. During the transition from PPB to spindle, the microtubules become both more closely associated with the nuclear envelope and aligned parallel with the PPB. Mitosis occurs, usually on days 3 or 4, within a relatively small portion of the
phragmosome, sometimes displaced from the centre of the cell. The phragmoplast then extends centrifugally through the phragmosome to fuse with the parental walls at the previously ordained division site. Cell plate maturation, on the other hand, appears to take place centripetally. The vast majority of the walls formed at the first round of division is periclinal, i.e., parallel to the wound surface.

A considerable amount of cellulose deposition also occurs in the lead-up to the first round of division. This deposition is related to the change in polarity brought about by (conventional) longitudinal wounding, as it does not occur in response to transverse wounding. In this regard, deposition and traumatotaxis are spatially coincidental, both occurring several layers into the wound, although subsequent divisions are confined to a much more limited zone.

The post-wounding deposition is polylamellate in nature with alternating layers of mainly transversely- and longitudinally-aligned cellulose. Deposition occurs on all the walls of the cell. It is greatest in the radial walls, less in the outer periclinal, and least of all in the inner periclinal wall. Marked deposition also occurs on the end walls. Hoops of cortical microtubules and congruent microfibrils are strongly implicated in the control of the post-wounding deposition, as they are in the unwounded condition, with microtubule patterns mirroring those of the adjacent wall. The layers of deposition are continuous from one wall face to another, both around the side walls and from the side to the end walls. Although the overall thickness of the layers varied within different walls, the total number of layers in each appears to be the same or very similar. These data point to a total-cell mode of deposition rather than, for example, the type found in the *Azolla* root, where individual faces have distinct cortical microtubule patterns - and
therefore patterns of cellulose deposition – that may be controlled by MTOCs at their edges (see Busby and Gunning, 1983). In the present system, differences in microtubule density between different faces could be related to changes in thickness of the individual layers around the cell.

The polylamellate structure of the deposition is most simply attributed to a wound-induced switch from a transverse to a longitudinal hoop of cortical microtubules. It is hypothesised that this alteration is initiated when the new stress pattern (after wounding) changes the 'free-energy' of the cortical array (sensu Green, 1984) so that a longitudinal, rather than a transverse hoop is more stable. Once in position, the longitudinal hoop would remain in place until the system is restabilised (after mitosis) and the free-energy barrier for a return to transverse arrays is overcome. It is hypothesised that the position of the longitudinal hoop rotates about the cell's long axis from one pair of opposite side walls to another, although encompassing the end walls at all times. The change from one set of wall faces to another is fairly rapid, as shown by the abrupt and not gradual changes in the orientation of the layers in the end walls. It is not certain whether the switching of the hoops from one wall face to another occurs via rotation of the structure or by disassembly and reassembly of the constituent microtubules. However, the former appears more likely in view of the fact that no cells with disassembled cortical microtubules were found. All day 1 and day 2 cells observed, by EM, possessed cortical microtubules, whilst only one cell not identifiable as mitotic was without them at days 3 and 4. This exceptional cell could well have been undergoing mitosis, but the nucleus was not encountered in the sections examined. The idea that there is a rotating longitudinal hoop of cortical microtubules, accords well with the deposition pattern in the end walls, and the spillover from the side to the
end walls. It is postulated that transverse layers in the side walls develop when cell the microtubules against faces not encompassed by the longitudinal hoop revert to a stable 'memory' position - i.e., are transversely aligned. Such a 'memory' condition has been implicated in many systems, not least in the general phenomenon of the reinstatement of transverse cortical arrays following mitosis (Green and Poethig, 1982) and in cells recovering from colchicine treatment (Taiz et al., 1981). All observations made so far of the distribution of cortical microtubules in pith cells of wounded D. stramonium pith are consistent with the above hypothesis relating the control of the pattern of the post-wounding wall deposition to the dynamics of the cortical microtubule arrays.

The mirroring of deposition patterns that often occurred in apposed walls of adjacent cells may be taken to implicate some form of supracellular control. In this respect, radial walls sometimes appeared to have a 45° layer interspersed between the otherwise orthogonal lamellae in the later-deposited layers and this could be the result of a partial breakdown in cell-to-cell control. A similar link between cells may be inferred from the observation that systrophic strands, phragmosomes and new cell-walls often line-up in 4-way junctions. Alternatively, this lining-up could, as suggested by Sinnott and Bloch (1941b), paradoxically reflect a breakdown in the cell-to-cell communications which, they suggest, usually causes a 'breaking of joints' at such sites. While a common feature of wound-induced divisions (e.g., Sinnott and Bloch, 1941a, b), 4-way junctions are normally, but not always (see Gunning, 1982b), avoided by the plant during normal development. Were the cells responding to a global stress pattern within the tissue when determining their division site, it seems reasonable that adjacent walls would line up.
After the first wound-induced cytokinesis, subsequent rounds of division occur rapidly, repeating the cytoplasmic events of star and phragmosome formation, but without traumatotaxis. Again, most of the divisions are parallel with the wound, but in the later rounds, more anticlinal and oblique walls are laid down. Mitoses are generally confined to a tier of cells between 2 to 4 deep. These are not immediately under the cut surface, however, as cells directly under the wound collapse and die soon after cutting, although some reach the stages of clustering of organelles and traumatotaxis before they succumb. The dead layer is usually one or two cells deep, but occasionally deeper. Immediately under the dead cells, a monolayer of rigid cells was sometimes present in which suberisation of the outer wall had occurred.

In the dividing zone, considerable radial expansion and thinning takes place in the radial walls of the mother cells, but it is not of sufficient magnitude to maintain average cell size, which drops markedly in all dimensions as division progresses. This is one of the relatively few examples of reduction in cell size accompanying division (Green, 1976). Expansion of the mother cells is largely radial, but the wound also expands laterally to some degree, as well as directly outwards. The expansion characteristics of the wound as a whole thus match the amount of post-wounding deposition in the individual walls of the responding cells.

As the walls thin, the tendency for 4-way junctions to form decreases, although many still occur. As the wound cambium develops, the 4-way junctions become sites of novel intercellular spaces. This lining-up of four walls could be a pre-requisite for intercellular space formation as, unlike their presumed normal ontogeny (see above), longitudinally-aligned microfibrils do not occur at the interior of such sites. The expansion constraints at 4-way junctions may be expected to lead to a localised
tearing upon expansion leading to formation of spaces (see also Sinnott and Bloch, 1941b).

Overall, it can be seen that the stimulation of the large, vacuolated cells of *D. stramonium* pith into dividing in a predictable fashion, allows the study of various factors related to the control of cell morphogenesis is general. Many of these aspects, such as phragmosomes and systrophes, would have been harder to observe in the small, densely-packed, cells of more normally-studied meristems. Additionally, in the present system, the lack of expansion of the cells until after cytokinesis, allowed the investigation of polylamellate wall structure without the problems of interpretation caused by wall extension during deposition.

In Chapter 2 (Section 2.4) it was proposed that the period from wounding, up to the first round of division, could be termed the 'primary re-differentiation phase', owing to the striking protoplasmic and wall reorganisation that occurs during that period (this provides further evidence - see also HARDHAM and McCULLY, 1982a, b - of differentiated plant cells not requiring an intervening mitosis in order to undergo an organised change of structure or function). Once primary re-differentiation has taken place, the cells divide rapidly and repeatedly. Such cells have been termed 'de-differentiated' (literature in WAREING, 1978). However, as the cells involved are meristematic they may be considered to be in a specialised form (Wareing, 1978), thus, it may be inappropriate to refer to them as de-differentiated, as this term implies a lack of specialisation. Furthermore, some cells maintain characteristics that would cause them to be defined as differentiated in the conventional sense. For example, in the current system, cells containing calcium oxalate that lie within the meristematic area divide to form progeny that also contain such crystals. Similarly, BLOCH (1948) noted that idioblastic
tannin-containing cells of *Ricinus* divide several times in response to wounding and all the daughter cells remain so characterised. However, although the term 'de-differentiation' seems inappropriate for the cells in the second phase of the wound response, a more apposite alternative is not immediately apparent. The term 'division phase' has been used (Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1983), but it could be more appropriate to continue attaching the prefix 're-' to differentiation when referring to an organised change of state, thus describing it as 'secondary re-differentiation'. If this is the case, when the cells re-differentiate again during the third phase of the response concomitant with cambium formation (Warren Wilson and Grange, 1984), the expression 'tertiary re-differentiation' would be consistent. The phases of the wound response in *D. stramonium* could then be summarised as follows:

![Diagram](image_url)

Several novel techniques were used to study the wound response. SM has been little used in plant studies, although the similar JB4 resin
(Polysciences) has been utilised in a number of studies, especially immunocytochemical ones (e.g., Franklin and Martin, 1980; Howlett et al., 1981). One of the main advantages of both SM and JB4 is that, unlike GMA, they do not require purification to remove the free methacrylic acid, which binds heavily to stains such as toluidine blue that contain basic groups (see O'Brien and McCully, 1981). Such purification techniques (e.g., Bennett et al., 1976) have, until recently, been very time-consuming and inefficient. Quicker methods have been developed (Franklin et al., 1981; Frater, 1979, 1981), but these are not free from problems (observations not described in this thesis; Frater, 1981). Another advantage of SM, with regard to immunocytochemical studies, is that polymerisation does not require UV radiation, which can cause loss of antigenicity (see Howlett et al., 1981). Nevertheless, in the present work this point proved to be academic because actin antigenicity appeared not to survive embedding per se (also Craig, 1984). Despite this, a partial study of the role of F-actin in the cytoplasmic wound response was made possible by the use of the CC MABs and NBD-Ph on hand sections.

Hand sections, but of a different nature, were also used, with polarisation and interference optics, to examine spatial differences in the response to wounding in both radial and end walls from different cells. This work was extended to observe longitudinal and end walls from the same cell by cutting-open isolated cells. Tissue maceration using EGTA or ethylenediaminetetraacetic acid was developed a number of years ago (Letham, 1960) to supplement older, more drastic, procedures for separating plant cells (e.g., see Tupper-Carey and Priestley, 1924). Similarly, large, single algal cells, such as those of Nitella internodes, have long been cut open for examination using polarising and interference optics (e.g., Chen, 1980, 1982; Gertel and Green, 1977; Green, 1958, 1960). As
far as the author is able to ascertain, isolated cells obtained from macerated interior tissue of higher plants have not previously been dissected to obtain single thickness, flat specimens of the whole cell for optical analysis.

This technique not only allowed detailed polarising and interference optical examination of the cells, in order to determine initial and post-wounding cell architecture, but also permitted development of a (still somewhat imperfect) method for immunofluorescence, that did not require enzymic wall degradation to secure antibody penetration. Opened-out walls also provided specimens for preparation of replicas and sections. However, in most cases the replicas showed very little surface relief, presumably owing to the incomplete extraction of the matrix substances. This was the case for a large number of shadowing angles and directions as well as replicas of varied thickness and composition (experiments not described in this thesis). Similarly, using high resolution SEM, little information was obtained about opened-out cells, either after fixation, incorporating T-X in order to try and visualise the cortical microtubules, or after cytoplasmic removal, in order to attempt to reveal the orientations of the innermost layer of microfibrils (experiments not described in this thesis). More vigorous extraction of the matrix substances may enhance surface relief. However, electron microscopic investigations of cells processed with fixative containing high concentrations of T-X showed that the innermost layers of microfibrils were readily disarranged so that a true picture of their orientation could be difficult to obtain. The innermost wall layer also binds pectin stains more heavily than older parts of the wall, suggesting that differences in composition may occur. The matrix substances may become more consolidated further into the wall.

In principle, a synthesis of these techniques should permit a fairly
detailed analysis of wall architecture and of cortical microtubule
distribution versus microfibril deposition direction to be made in a single
cell, *viz.*: immunofluorescence (anti-tubulin) examination of all the walls
of an opened-out cell; polarisation and interference study of the same;
cutting the specimen in half transversely; one portion of side wall and one
end wall then embedded and sectioned for examination of wall structure
using EM, and the remainder of the walls used for production of a
replica. However, it is clear that the techniques would need to be refined
before such an approach could be successful.

Other experiments could also be performed in order to extend the work
described in this thesis and thus gain a better understanding of the
factors involved in the wound response and their relation to plant
cytomorphogenesis in general. Further studies on the role of stress in
determining the plane of division would perhaps be most easily carried
out. The recently-matured internode that is used for wounding is still
fairly flexible and, if not supported after wounding, occasionally bends.
Deliberate bending of the wound, either immediately after wounding or at
intervals thereafter, would be expected to change the stress-patterns
within the tissue (cf. Yeoman and Brown, 1971). Curving the wound convexly
to an angle of 90° would be expected to produce longitudinal forces of
tension in the sub-wound area. If Lintilhac's 'rules' are followed (e.g.,
Lintilhac, 1983) cell plates will form in shear-free planes so that the
plane of division would be expected to change from periclinal in the normal
wound to transverse (anticlinal) in the bent wound. A similar result would
be expected if the wound was bent concavely (giving forces of compression),
but such an arrangement would be harder to achieve. Relacing the removed
tissue - possibly after fixing it - at varying intervals of time after
wounding and securing it firmly in place (in the manner of Brown and Sax,
1962), might also reveal something about the role of stress (as pressure) in the wound response. However, caution should be exercised when interpreting the data obtained from experiments that involve the covering of the surface of the wound. It has been shown that sealing the wound in *D. stramonium* with 'Vaseline' or lanolin, at various times after wounding, causes changes in the final pattern of tissue differentiation (Warren Wilson and Warren Wilson, 1961a, 1983). It would be necessary to investigate the cytoplasmic and wall changes that are associated with any shift in the division direction that may result from the modification of the stress-patterns within the wound.

Such experiments may help determine the critical period for stress detection by the cells. For example, if systrophes are responsible for determining the plane of division, bending treatments imposed immediately after wounding and removed at day 1, would not be expected to change the orientation of the new cell walls.

Another large area of work which could be carried out concerns the effects of drug treatments upon the wound response. However, caution must be exercised when interpreting the data obtained from such experiments, as compounds such as colchicine may effect several cellular processes (e.g., Hart and Sabnis, 1976). Furthermore, it is not immediately apparent how drug solutions could be applied and limited to the wound area, although Evans (1979) has used a syringe-needle drip system in an attempt to determine the effects of added auxin on regeneration patterns in split hypocotyls of *H. annuus*.

As discussed in Chapter 4 the use of anti-actin drugs such as cytochalasin B would be of especial interest. Such a treatment prevents traumatotaxis in *Tradescantia* leaflets (Schnepf and von Traitteur, 1973) and, in view of the presence of actin filaments around enclustered and
traumatotactic nuclei in the current system, may be expected to prevent nuclear migration in D. stramonium. If inhibition occurs, the requirement for traumatotaxis, as a pre-requisite for post-wound deposition, could be assessed (cf. transverse wounding where neither occur). Cytochalasin or similar treatments may also illuminate the role of F-actin in systrophic and phragmosomal formation and, perhaps, in the organisation of the division site.

The effects of anti-microtubule drugs on the cytoplasmic reorganisation could also be enlightening. However, such treatments would probably be most informative about the control exercised by cortical microtubules over the post-wounding wall deposition. From studies on other systems, two responses seem likely: either the polylamellate deposition will become random (cf. Green, 1963) or it will continue in one direction only rather than switching orientations (cf. Robinson and Herzog, 1977). In the latter case, the anti-microtubule drug would need to be applied after the post-wounding deposition had started (day 1). Similarly, the effects of ethylene upon the post-wounding deposition would be of interest. This substance is generally described as changing microfibril deposition and cortical microtubules from transverse to longitudinal (e.g., Ridge, 1973; Steen and Chadwick, 1981), although this is disputed (e.g., Reis et al., 1982). The effects are not clear cut in polylamellate systems, but polylamellations still remained in cells from ethylene-treated epicotyls of Pisum sativum (Lang et al., 1982) and hypocotyls of Vigna radiata (Reis et al., 1982; Vian et al., 1982).

Also required in order that a more complete picture of events after wounding can be obtained is a detailed examination of deposition after the first round of division. As considerable expansion and thinning occurs in the mother-cell walls, it is difficult to determine if any deposition takes
place at such faces. However, many walls arise de novo, especially during the later rounds of division. It would be of interest to determine the nature of these walls, especially if they are polylamellate and, if they are not, to determine the relationship between their direction of cellulose reinforcement to their orientation within the wound and their likely direction of expansion. Superimposed on this would, of course, be a study of the role of cortical microtubules in the control of microfibril deposition direction.

A general ultrastructural examination of the later divisions may enable structures associated with subsequent cambium formation to be recognised. In this regard, a study of the response of the outer cortex could serve as a useful comparison. These cells do not form a cambium, although they do undergo some wound-induced divisions parallel to the wound, and preliminary studies (not described in this thesis) indicate that they show the protoplasmic reorganisation and wall-thickening observed in the pith cells.

The ultrastructure of the initial stages of the wound response of parenchyma cells in dicotyledonous stems does not appear to have been studied in detail. The question arises as to whether the events observed in the current study are purely a phenomenon of \textit{D. stramonium} or whether they are of widespread occurrence. The latter seems to be by far the more likely, as the large-scale anatomical changes observed in \textit{D. stramonium} have been described in many other dicotyledonous species (see Warren Wilson and Grange, 1984; Warren Wilson and Warren Wilson, 1983). Furthermore, initial light and electron microscopic studies (not described in this thesis) of wounded internodes of \textit{Helianthus annuus}, which show tissue regeneration typical of dicotyledonous stems (Ms M.J. Hurley, Botany Dept., A.N.U., \textit{pers. comm.}; Warren Wilson and Warren Wilson, 1983), demonstrate the same pattern of events observed for \textit{D. stramonium} - notably the protoplasmic reorganisation and polylamellate wall deposition.
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