Hypocotyl Growth During Seed Germination In *Lupinus angustifolius*

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ABSTRACT

Hypocotyl growth during seed germination in Lupinus angustifolius cv. New Zealand Bitter Blue was studied.

There was an initial period of rapid hypocotyl elongation over the whole length of the hypocotyl from 0.5 to 1.5 days. Cell division in the cortex occurred between 1 and 3 days, with a peak at 1.5 days. Total hypocotyl length increased rapidly from 2 to 4 days. The distribution of regions of elongation within the hypocotyl changed during this expansion phase, with a wave of cell elongation proceeding acropetally from the base.

The embryo hypocotyl in the seed contained 50 % of the maximum amount of RNA found in the germinating hypocotyl. The peak rate of RNA synthesis for the hypocotyl as a whole, occurred at 12 hours. RNA content of whole hypocotyls doubled between 0.5 and 1.5 days. Nucleoli reached their maximum size within the first day of germination. The loss of vacuoles and granular zones accounted for the subsequent decrease in nucleolar size. At the time of maximum nucleolar size, the nucleus was often convoluted and the nuclear membrane had a high frequency of pores. Cytoplasm was densely packed with polyribosomes and rough e.r. until the cells started their rapid elongation phase at approximately 2 days. RNA synthesis declined rapidly after this time.

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CHAPTER ONE INTRODUCTION

In the preface to the publication of the Proceedings of the Sixth International Conference on Plant Growth Substances, Wightman and Setterfield (1968) state that for detailed understanding of developmental processes, complex systems involving integration of events at the molecular, cell, tissue, organ and organism levels will have to be invoked.

In this project an integrative approach to the study of development of the hypocotyl of lupin (Lupinus angustifolius) is used. The hypocotyl is described at the levels of its morphology, cellular events, subcellular organelles, and molecular content (specifically RNA). The characteristics of the hypocotyl are monitored from the time of seed germination until the seedling is five days old.

In recent years the electron microscope has been used extensively to study ultrastructure of cells at various stages of development. However, such studies have been concentrated on either meristematic cells or mature cells which are engaged in a specialized function. Relatively few workers have made a comparative electron microscope study of dividing, elongating and maturing cells, particularly in non-specialized tissue such as cortex parenchyma. Even a recently published comprehensive book on plant ultrastructure (Robards, 1974), which covers a wide range of organelles and specialized cell types, does not contain a chapter on general formation and development of parenchyma cells.

Although cellular growth from division to maturation is often divided into stages, it is actually a dynamic continuum of events. A sigmoid curve describes the increase in length of an expanding cell with time. The cell elongation stage may be divided into the early exponential

phase and the later linear phase. In the early phase the relative growth rate of a cell is high but the actual increase in length is low. Conversely, in the second phase, the actual increase in length is high but the relative growth rate is lower than in the early phase.

Elongating cells, especially in the second phase of elongation, have not been extensively studied under the electron microscope. This may be the result of the technical difficulties of satisfactorily fixing the highly vacuolated cells that are typical of this stage of development. And also, the thin layer of cytoplasm initially looks to be of little interest compared with the contents of meristematic or specialized mature cells.

Several comparative electron microscope studies of developing plant tissue have been made. The tissues studied include root apices of Plantago ovata (Hyde 1967), Allium cepa (Chounard 1975) and Pisum sativum (Chaly and Setterfield 1975), the wheat coleoptile (Rose 1974) and reactivating storage tissue of Jerusalem artichoke (Fowke and Setterfield 1968, Jordan and Chapman 1971). These electron microscope studies complement analytical and light microscope studies of such tissues and, together with the many other approaches to the study of cell development, will help in forming an understanding of the general pattern of growth in plants.

Information on the developmental process has resulted from several different approaches to its study. In this particular laboratory , the role of auxin in elongation of the hypocotyl of light-grown lupin seedlings has been studied. Penny (1969) found that there is a lag phase of about 15 minutes before auxin stimulated elongation occurs, which suggests that cell elongation is not the primary action of auxin. An understanding of the physiological processes in expanding cells may be aided by an electron microscope study of the region. Penny (1971) did an electron

microscope study of the rapidly elongating zone of a 4 day old lupin hypocotyl and described a typical expanding non-vascular cell as being highly vacuolated and containing only a few golgi and free ribosomes but frequent rough endoplasmic reticuli. Paramural bodies were also seen commonly. These are membranous structures associated with the cell wall and are postulated to be involved in cell wall synthesis (Marchant and Robards 1968).

It has been found also, that RNA synthesis is not necessary, at least initially, for the action of auxin on elongation (Penny P. et al. 1972). Kinetic studies were made on the effect of Actinomycin D, an RNA synthesis inhibitor, on auxin induced growth. Auxin induced growth continued the same in tissue pretreated for up to 2 hours in Actinomycin D and remaining in Actinomycin D during treatment with indole acetic acid (IAA), as it did in control tissue treated with IAA only. The growth rate in inhibitor-treated tissue dropped below that of the control about 2 hours after addition of IAA. These results suggest that RNA synthesis is not recessary for the initial action of auxin on elongation.

There is a lot of evidence to suggest that RNA synthsis in its various forms has a central role to play in developmental processes in plants. It is important in the chain of physiological events between the hormonal stimulus and the cellular response. Several approaches to the study of the role of RNA in developmental processes have been used and, because of their relevance to this project, some of these results are reported here.

One approach has been to determine the absolute amount of RNA present in tissue at different times or at a range of distances from a meristematic apex, and also to determine the actual time when RNA synthesis is occurring in relation to the stage of development of the tissue.

Heyes (1960) studied the root tip of Pisum sativum and found that

RNA content per cell increased from 4×10^{-5} µg per cell at 1mm from the tip to 16×10^{-5} µg per cell in mature cells 8-10 mm from the tip.

In the coleoptile of germinating wheat, Wright (1961) found that the period of rapid cell division is from 24 to 48 hours and the most rapid cell expansion from 48 to 84 hours. On a per coleoptile basis, the increase in RNA from 24 to 48 hours was 275% and from 48 to 72 hours was 31%. This indicates that the rate of RNA synthesis is much higher during the cell division than cell expansion stage.

Giles and Myers (1964) studied growth of the hypocotyl of Lupinus albus. They report that, from germination to 14 days, growth is purely by cell expansion, all cell division having occurred during formation of the seed. (Buis, 1967, reports the presence of some cell division up to 5 or 6 days in hypocotyls of the same species.) A period of rapid increase in RNA content of the hypocotyl precedes the period of rapid increase in hypocotyl length. In the dark the hypocotyl expands to about 20 cm in 14 days compared with about 5cm in continuous light. The maximum RNA content of the dark grown hypocotyl is twice as great as for the light grown one. Van Oostveldt et al. (1976) found, similarly, that light strongly inhibits growth of the hypocotyl in Lupinus angustifolius and also causes a reduction in synthesis of RNA.

Chaly and Setterfield (1975) used autoradiographic methods to determine the time of RNA synthsis relative to the stage of development of the tissue. They found that incorporation of ^{3}H-cytidine into RNA is very high in cells from 0 to 1mm from the root tip of Pisum sativum, but decreases exponentially from 2mm basipetally. There is still a small amount of incorporation up to 7 mm from the tip.

Rose (1974) studied incorporation of ^{3}H-uridine into the nucloli of germinating wheat coleoptile cells. The nucleoli in tissue excised

at 15 hours show the highest incorporation, while the nucleoli from tissue excised at 73 hours show very little incorporation of the labelled uridine. The time of highest incorporation slightly precedes the time of initiation of cell division.

These studies indicate that RNA synthesis is most rapid either during or slightly preceding the cell division phase in the tissue and declines very rapidly in elongating and maturing tissue.

A second and very informative approach to the investigation of the role of RNA in developmental processes is electron microscope study of the nucleolus. A lot has been learned from studies of nucleoli of cells at various stages of development.

The nucleolus of cells in tissue prepared for electron microscopy exhibits three zones. These are described by Jordan and Chapman (1971). The granular zone is made up of 15 nm densely staining granules. The fibrillar zone is composed of densely packed fibrils measuring about 10 nm in diameter. The third region appears under the electron microscope as lightly staining vacuoles within the nucleolus. This region is considered by Jordan and Chapman (1971) to be the nucleolar-organizing region of the nucleolar chromosome. That is, a part of the nucleolar chromosome which has expanded so that it can function as the template on which ribosomal RNA is synthesized. Jordan and Chapman regard the change in distribution of the vacuolar region as fundamental and the other nucleolar changes as being consequent upon it.

An important study was made by Fowke and Setterfield (1968). They used electron microscope techniques to compare the ultrastructure of cells from dormant Jerusalem artichoke tuber with cells from tuber tissue which has been "aged" by being cut in thin slices and placed in aerated water

at 25°C. Observations relevant to the nucleolus are mentioned here. In cells fixed directly from the intact tuber, nucleoli were dense, homogeneous and fibrillar. There were no vacuolar or granular zones. Nucleoli in "aged" tissue showed distinct granular and fibrillar zones, and vacuoles within the nucleolus were common. Fowke and Setterfield showed that nucleolar size increases during aging of discs of Jerusalem artichoke. Ultrastructural changes can be related to the changes in nucleolar size. Nucleoli of dormant tuber cells appeared homogeneous and compact and were small. In cells "aged" for 24 hours the nucleoli were enlarged and showed distinct fibrillar and granular zones. Vacuoles were found within both granular and fibrillar zones. Chapman and Jordan (1971) have suggested that nucleolar size is directly related to nucleolar activity. This generalization seems to be true in many cases but Chaly and Setterfield (1975) warn that this size-activity correlation may be misleading. In Pisum sativum roots cut into ten 1 mm segments basipetally, nucleoli 1 mm and 3 mm from the apex were very similar in size, as were nucleoli 5, 7 and 9 mm from the apex, but according to autoradiographic data, segments 1 and 3 incorporated ^{3}H-cytidine at very different rates, as did nucleoli of segments 5 and 9. Also the nucleoli differed in ultrastructure within each group.

These studies show that marked changes in the ultrastructure of the nucleolus accompany changes in the level of RNA synthetic activity.

The third approach to studying the role of RNA in developing plant tissue has been to determine the effect of 5-fluorouracil (5-FU) on RNA metabolism. 5-FU is a pyrimidine analogue which selectively disrupts synthesis of ribosomal and transfer RNA without significantly influencing synthesis of messenger RNA (Key and Ingle 1964).

Masuda, Setterfield and Bayley (1966) found that 5-FU failed to inhibit the rapid expansion growth of either intact coleoptiles or isolated coleoptile segments. Setterfield (1963) found that 5-FU given during the "aging" period inhibited subsequent auxin-induced elongation in Jerusalem artichoke tuber slices and Fowke and Setterfield (1968) have shown by electron microscope studies that ribosomes are synthesized in this aging period. Rose and Setterfield (1971) found that RNA precursors were incorporated into the nucleolus during 5-FU treatment but that label did not appear in the cytoplasm. Such nucleoli were found to lack a distinct granular zone and appeared as homogeneous fibrillar structures under the electron microscope. King and Chapman (1972) found that when discs are aged in 5-FU, the nucleolar volume increases 50 to 70 % more than control tissue, which suggests that 5-FU causes accumulation of ribosomal-RNA precursors within the nucleolus.

These results suggest that the rapid elongation phase of growth depends on the formation of ribosomes at an earlier stage. The effect of 5-FU is probably to disrupt the actual assembly of ribosomes or their transport from the nucleolus, since RNA precursors are still incorporated and appear to be accumulated in the nucleolus and no granular zone forms.

The fourth approach has been to determine the level of RNA polymerases in tissue at various stages of development. At least one enzyme (RNA polymerase I) is compartmentalized within the nucleolus and is presumably involved in synthesis of ribosomal RNA <u>in vivo</u>. There is also at least one polymerase (RNA polymerase II) in the non-nucleolar nucleoplasm. It is involved in the formation of messenger RNA from the DNA template.

Mature hypocotyl of soybean, when treated with 2,4-D, becomes

swollen and proliferates abnormally. In such tissue Guilfoyle et al. (1975) found a 5 to 8 fold increase in the amount of Polymerase I. The level of Polymerase II remained similar to that in the untreated control.

Rizzo and Cherry (1975) measured the relative levels of RNA polymerases in soybean hypocotyls during various stages of development. The meristematic region has more total polymerase activity per gram fresh weight and a greater proportion of Polymerase I to Polymerase II than the differentiated regions. The fully elongated tissue contains mainly Polymerase II.

The level of RNA polymerase I in the nucleolus gives an indication of the amount of ribosomal-RNA synthesis occurring. There is more to be learned from studies like that of Rizzo and Cherry in which the level of RNA polymerase is correlated with either the stage of development of the tissue or with the ultrastructure of the nucleolus.

The fifth approach to the study of the central role of RNA in development is to follow the changes in density of ribosomes either per cell or per volume of cytoplasm, as tissue passes through the developmental stages.

Chaly and Setterfield (1975) covered this in their study of pea roots cut into 1 mm segments basipetally. The cytoplasm of segment one was packed with free ribosomes, and only isolated elements of rough endoplasmic reticuli (e.r.) were present. By segment 3, extensive rough e.r. had formed and this persisted through to segment 9. Segments 3 to 7 had the highest density of ribosomes on e.r. In the non-growing segments, 7 and 9, the free ribosome population appeared greatly reduced. The density of membrane-bound ribosomes and the amount of e.r. per wall length also decreased.

Hsiao (1970) estimated the number of ribosomes per cell in 3 regions of the root tip of Zea mays. In the apical region, 0 to 3 mm, there were 17 x 10^6 ribosomes per cell. The regions 6 to 12 mm and 12 to 40 mm both had about 25 x 10^6 ribosomes per cell.

Jordan and Chapman (1971) report an increase in ribosomes and rough e.r. in the cytoplasm after 24 hours aging of carrot discs.

Masuda et al. (1966) found the concentration of both free and bound ribosomes does not increase in growing oat coleoptiles and may even decline. Free ribosomes decline markedly in fully grown coleoptiles, while the proportion of bound ribosomes increases. They conclude that young coleoptiles contain a full complement of ribosomes necessary for subsequent growth.

Tata (1971) suggests that the association of ribosomes with e.r. may function to segregate different populations of ribosomes synthesizing different classes of proteins.

In summary, these five approaches lead to the general conclusion that RNA synthesis is greatest in meristematic and early elongating tissue and that during this time the number of ribosomes formed is sufficient to sustain subsequent expansion growth. It is the aim of this project to study this generalization in lupin hypocotyl tissue and find how it relates to other processes at the molecular, subcellular, cellular and tissue levels.

Lupin, Lupinus angustifolius cv New Zealand Bitter Blue, was chosen as the study species because it is used in physiological experiments, particularly in this laboratory. Therefore a description of the growth of the plant at cellular and subcellular levels may be useful for

hypothesizing a mechanism to explain a physiological response in an experimental situation.

Other advantages of lupin, which were found after the study had begun, were that cells of the hypocotyl show two phases of elongation. Presumably, certain cytological events are common to the process at both stages. Other commonly studied systems contain a meristematic apex from which cells arise and show one phase of elongation before maturing. In the hypocotyl of lupin the initial phase of expansion is by cells which have been in an inactive state for a variable time period in the seed, whereas, in say a root, it is active meristematic cells which elongate.

The epidermal cells of lupin were found not to divide after germination, so the cytological events which occur in these cells at an early stage are required for elongation. Early events in cortical cells may be required for cell division as well as elongation.

In this study the growth of the hypocotyl is defined by its increase in total length over the growth period. Then growth is explained in terms of the contributing factors at the cellular level; cell division and cell elongation. By knowing the distribution of these cellular events in the hypocotyl, tissue can later be taken from a region whose cellular activities are known, and used for analyses of cellular contents. This will enable correlations to be made between cellular activity and cell contents. This type of correlation has often been made for cells with a specialized function, but not so often for unspecialized cells such as cortex parenchyma.

At the sub-cellular level this study concentrates on the formation of ribosomal RNA in the nucleolus and its presence in the cytoplasm. This is considered to give an indication of protein synthetic activity in the cell.

Cells at a known stage of development are studied at the subcellular level. The first approach is a light microscope study of the nucleolus, measuring its size and noting the extent of nucleolar vacuoles. Secondly, electron microscope techniques are learned and used to study the ultrastructure of the nucleolus at various stages, and also the density and distribution of ribosomes in the cytoplasm. The third approach is to chemically isolate ribosomal RNA and determine the relative amounts in cells at different stages of development. Similar methods are used to determine the stage at which RNA synthesis is most rapidly occurring.

As well as the RNA-associated organelles, description of other organelles is included in the hope that they will help in forming a fuller, more general description of plant growth, in conjunction with other more experimental approaches.

By using several approaches to the study of the lupin hypocotyl, a wide range of experimental techniques will be learnt and used.

CHAPTER TWO MATERIALS AND METHODS

2.1 Growth of Seedlings

Lupin seeds, Lupinus angustifolius cv New Zealand Bitter Blue, were surface sterilized in 1% sodium hypochlorite bleach for 15 minutes. They were then soaked in running tap-water (about 15°C) for 2 hours, following which they were planted in moist vermiculite. Seeds were orientated in the vermiculite with the embryo hypocotyl perpendicular to the surface of the vermiculite. Pots were placed under continuous light in a growth room. The temperature at the surface of the vermiculite ranged from 23°C to 25°C. The light intensity at the surface of the pot was 50 μ Einstein m^{-2} sec^{-1} supplied by a combination of 84% fluorescent (Phillips TLA 80W/55) and 16% incandescent light. The pots were standing in a 1 cm depth of water.

2.2 Measuring Total Length of Hypocotyl

Fourteen pots, each with 50 seeds, were treated as described above. At 12-hourly intervals after the time of planting, one pot was removed and the ten most vigorous seedlings were selected and the length of their hypocotyls measured. With the very young seedlings, measurement was made with the aid of a calibrated micrometer eyepeice in an Olympus stereo microscope. The mean hypocotyl length of 10 plants at each age was determined.

2.3 Measuring Hypocotyl Diameter

Hypocotyl diameter was measured within 0.1 mm using a calibrated micrometer eyepiece (100 divisions = 1 cm) in an Olympus stereo microscope. The diameter was measured at three positions on the hypocotyl; at the

base, at the top directly below the cotyledons, and at a mid-point on the hypocotyl. At each position both the widest and narrowest diameters were measured. The mean diameter was determined by averaging the six measurements over the ten seedlings at each age.

2.4 Measuring Regions of Elongation

A row of ten seeds was planted in each of two pots with a 12 hour time lag between planting the seeds in each pot. At 24 hours after planting, 4 seedlings from each pot were selected and the testas removed. Marks were placed at approximately 1 mm intervals on the hypocotyl which, at this age, is about 10 mm long. Marks were made by applying a warm paste of charcoal in lanolin with a fine-tipped needle. Seedlings were photographed immediately after marking. In all photographs a metric ruler was included, and was placed in the same plane as the seedlings. They were lightly re-covered with vermiculite then watered. The charcoal-lanolin markings are not removed by watering. Seedlings were photographed at 4 hourly intervals between 10 am and 10 pm daily until they were 3 days old. Because of the 12 hour age-gap between seedlings of the two pots, elongation was effectively measured 4 hourly between 1 and 3 days. Negatives were developed then viewed under an Olympus stereo microscope. Plate 3.1 shows marked hypocotyls. A micrometer eyepiece was calibrated using the mm scale on the negative and the distance between marks was measured from each negative and recorded.

A new set of plants was used to determine the regions of elongation from 3 to 6 days but basically the same methods were used.

2.5 Measuring Cell Dimensions

Measurements were made on the plant material which had been fixed and

embedded for electron microscopy, as described in section 2.9. Longitudinal sections were cut on an LKB Pyrimatome with a glass knife. Sections were cut 5μm thick in the dense 16 hour and 1 day tissue, but all others were 10μm in thickness. They were placed on a drop of water and dried onto glass slides, stained in toluidine blue and mounted permanently with Eukitt mounting medium. Using light microscopy at a magnification of 400 times, cell length and width were measured with a calibrated eyepiece micrometer. Cells are arranged in longitudinal files in the tissue and 10 to 20 cells of a file of epidermal cells, outermost cortex cells, and inner cortex cells were measured at each age and position of hypocotyl sampled. Cell length was difficult to measure once cells were longer than about 200μm because sections rarely passed through the full length of a particular cell.

2.6 Cell Division Analysis

2.6.1 Staining and Sectioning

Hypocotyls of the required ages were fixed in fresh 3:1 ethanol: acetic acid for 24 hours then preserved in 70% ethanol. Sections 1 to 2 mm long were cut from the hypocotyl at each of the three defined regions at each age. These were stained by the Feulgen method as follows.

Rinse preserved section in distilled water

Hydrolise in 1 N HCl for 15 minutes at 60°C

Rinse in distilled water

Stain in Feulgen solution (appendix 3) in the dark at room temperature for 3 hours.

Rinse in distilled water

Bleach in Sulphur-dioxide water (appendix 3) two changes of 10 minutes each

Place in running tapwater for 30 minutes. (During this time the tissue

changes colour from light purple to intense red.)

During this procedure the tissue blocks were contained in sieve-bottomed plastic tubes. These were made from 2.5 cm lengths of 1 cm diameter rigid plastic tube with a piece of cloth mesh glued to the base with epoxy glue. Tubes were marked by cutting notches in the top. Tissue could therefore be transferred easily from one solution to another. The stained tissue was placed in gelatin fluid (appendix 3) for between 15 minutes and 2 hours before being sectioned on a Frigistor freezing microtome. The block of tissue was frozen onto the stage in gelatin fluid and 50 μm thick sections were cut. The anti-roll plate was adjusted to obtain flat sections. Sections, which adhered either to the knife or the plate, were picked up directly onto glass slides, about 18 sections per slide arranged serially. They were air dried then dehydrated rapidly through 70%, 95% and absolute ethanol. Slides were made permanent by mounting in Eukitt mounting medium.

2.6.2 Counting Cell Division

Slides were viewed on an Olympus compound microscope under 400 times magnification. A squared grid, with a total area of 1.56×10^{-2} mm^2 was placed in the eye-piece. With 50 μm sections the volume of tissue seen under the grid is 7.81×10^{-4} mm^3. For the vascular and cortex tissue at each selected age and position of the hypocotyl, the average number of nuclei per grid volume was determined. The number for each grid volume was fed directly into a programmable calculator. The mean, standard error of the mean (S.E.M.), and ratio of the S.E.M. to the mean, expressed as a percentage, could be calculated directly and further counts made and accumulated until the percentage error reached an acceptable level. This was set at 8% for tissue that was very variable but mostly levels below 5% were obtained. Once the number of cells per grid view was calculated, the

cells in division could be counted from a large number of sections. The total number of grid volumes viewed was counted on one click-counter and the number of cells in division on another. The number of nuclei in division per 1000 cells was calculated.

2.7 Measuring Nuclear Diameters

Sizes of nuclei from cells at different stages of development were not compared so it was considered adequate to measure diameters of nuclei prepared as for cell division analysis (section 2.6.1). Measurements were made using a calibrated eye-piece micrometer in an Olympus compound microscope.

2.8 Measuring Nucleolar Diameters

This was done on tissue prepared for measuring cell dimensions (section 2.5). Measurements were made by means of a Watson ocular micrometer (filar or moving micrometer eye-piece) equipped with a slidewire which was manually manipulated by revolving a wheel calibrated into 100 units. With the magnification used (15 x ocular, 100 x objective) each unit on the wheel was equal to 0.023 μm. The accuracy of measuring was ± 2 units which is ± 0.046 μm on each nucleolar measurement.

2.9 Techniques for Electron Microscopy

As a starting point, tissue was prepared for electron microscopy by a routine method used by the Electron Microscope Unit of the D.S.I.R., for plant and animal material.

The primary fixative is a modified Karnovsky fixative. It is 2% formaldehyde and 3% glutaraldehyde in a 0.1 M phosphate buffer at pH 7.2.

Sections are cut from a known position of the lupin hypocotyl. Using

a sharp razor blade, 1 mm segments are cut while the specimen is in primary fixative. The segments are placed in primary fixative in labelled glass vials then placed under a vacuum of 15 - 20 mm Hg for about 5 minutes at room temperature to infiltrate fixative into the intercellular spaces and also to remove bubbles trapped by the hairs on the outer surface of the hypocotyl. The primary fixative is then replaced incase any aldehydes have evaporated during the vacuum infiltration. The following procdure is then followed:

	Time	Temperature
Primary fixation		
Karnovsky fixative	2 hours	4°C
Phosphate buffer (pH 7.2) wash	1 rapid	4°C
	2 ½hour	4°C
Secondary fixation		
1% Os O_4 in Phosphate pH 7.2	3 hours	4°C
Buffer wash	1 rapid	room temperature
	1 ½hour	room temperature
Dehydration		
25% Acetone	½hour	room temperature
50% Acetone	½hour	room temperature
75% Acetone	½hour	room temperature
95% Acetone	½hour	room temperature
100% Acetone	½hour	room temperature
Infiltration		
25% resin in Acetone	2 hours	room temperature
50% resin in Acetone	3 hours	room temperature
75% resin in Acetone	overnight	room temperature
100% resin	6 hours	room temperature

Embedding
Embed in fresh resin in size 3 gelatin capsules. Resin is Durcupan ACM epoxy resin ("Fluka"). See appendix 1.

Curing
Place in a 60°C oven for 2 or 3 days.

Although the above procedure proved satisfactory, better results were obtained from the following procedure. Most of the micrographs shown are of tissue prepared in this way.

	Time	Temperature
Primary fixation		
3% glutaraldehyde in 0.025 M Na-Cacodylate buffer pH 6.8	3 hours	room temperature
Na-Cacodylate buffer wash	2 rapid	1°C
	overnight	1°C
Secondary fixation		
1% OsO_4 in Na-Cacodylate buffer pH 6.8	3½ hours	room temperature
Buffer wash	2 rapid	room temperature
	1 ½hour	room temperature
Dehydration		
25%%Acetone	½hour	room temperature
50% Acetone	½hour	room temperature
75% Acetone	½hour	room temperature
95% Acetone	½hour	room temperature
100% Acetone	½hour	room temperature
100% Acetone plus anhydrous $CuSO_4$ in a desiccator	½hour	room temperature
100% propylene oxide	1 rapid	room temperature
	2 hours on stirrer	
Infiltration		
25% resin in propylene oxide	2 hours	room temperature
50% resin in propylene oxide	overnight	room temperature
100% resin	6 hours	room temperature

Embedding

Embed in fresh resin in number 3 gelatin capsules (stored in desiccator) and cure in 60°C oven for 3 days.

Sections were cut with glass knives made on an LKB Knifemaker. The block was first shaped on an LKB Pyrimitome then ultrathin sections were cut on an LKB Ultratome. The collecting fluid in the boat on the knife was 5% ethanol solution. Light gold sections were cut and picked up unsupported on copper grids with a mesh size of 50 μm. Grids were rinsed in acetone and air dried prior to picking up sections which were adhered to the matt surface of the grids. Prepared grids were stored in a dust-proof grid-holder.

Prior to viewing in the transmission electron microscope, grids were double stained by the following method. Place grid in a drop of Uranyl Acetate (Appendix 1) for 6 minutes. Rinse in 50% Ethanol then distilled water. Place grid in a drop of Lead Citrate (Appendix 1)for 5 minutes.

Rinse thoroughly in distilled water.

2.10 Analysing Content and Synthesis of RNA in Hypocotyls

Hypocotyls from seedlings at ages 0.5, 1, 1.5, 2, 3, 4 and 5 days were used in determining both content and synthesis of RNA. The RNA content of embryo hypocotyls from dry seeds was also determined.

To determine rates of RNA synthesis at each age, excised hypocotyls were incubated in 0.02 M tris-maleate buffer at pH 6.1 in a 25°C shaking water bath for a 1 hour pretreatment period. This was followed by a further 1.5 hour incubation in fresh buffer solution containing about 0.2 μCi ^{32}P per treatment.

Hypocotyls up to the age of 1.5 days were incubated whole, but older hypocotyls were cut into 10 mm segments for incubation. At each age, three replicates each containing ten hypocotyls were weighed then incubated. Tissue was then rinsed briefly in two changes of distilled water before being frozen in liquid air. One of the three replicates was ground with a mortar and pestle and made up to a total volume of 10 ml with distilled water. The debris was spun down in a centrifuge and a 0.5 ml aliquot of the supernatant was taken for determination of total radioactivity in the tissue, using a Packard liquid scintillation counter.

RNA was extracted from the other two replicates using a method of tissue disruption followed by nucleoprotein dissociation and deproteinization of the released RNA (Stewart and Letham, 1973). Details of the method are given in appendix 2. The final RNA precipitate was dissolved in 5 ml of electrophoresis running buffer (see appendix 2). From this solution an aliquot was taken for scintillation counting to determine the counts incorporated into RNA. The remainder of the solution was used for a spectrophotometric determination of total RNA content of the hypocotyls.

A standard curve, showing the relative absorbance at 260 nm of RNA solutions at a range of known concentrations, was prepared. The standard solutions were made up with yeast RNA in the electrophoresis running buffer. This buffer was also used as a reference solution in the spectrophotometer.

An indication of the purity of the RNA extract is given by the ratio of absorbances at 260 nm and 280 nm, which should be about 2. Using this extraction method values between 1.8 and 2 were obtained.

In order to check that RNA synthesis was not greatly stimulated by the cutting and incubation of the tissue, a fourth set of 10 seedlings was taken from the same pot as the first three replicates at the time when the others were removed from incubation. The same RNA extraction procedure was used on these hypocotyls and it was found that the RNA content did not differ significantly from that of the incubated tissue so this was used as a third replicate for determination of RNA content.

CHAPTER THREE GROWTH OF THE LUPIN HYPOCOTYL

The morphological changes in the hypocotyl are described and explained in terms of the contributing developmental processes of cell division and cell elongation.

The absolute growth, or velocity, may be expressed as $\frac{dL}{dt}$ with units of length per time ($mm.hr^{-1}$), where L is length and t the time interval. The relative growth rate may be expressed as $\frac{dL}{L_o.dt}$ where L_o is the length at the start of the time interval t. This gives a ratio per time (hr^{-1}).

Green (1976) uses the formula $\frac{\ln L_1 - \ln L_o}{t}$ for calculation of relative rates of growth. The relative growth rate does not include a length unit because it is a ratio expressing the change in length of the segment over a defined time period compared with the length of that segment at the beginning of the time period.

3.1 Total Hypocotyl Growth

3.1.1 Hypocotyl Length

Figure 3.1 A shows the actual hypocotyl length of seedlings grown under the conditions described in section 2.1. The calculated standard errors of the mean are 2% or less of the mean for the 10 seedlings measured at each age. Green (1976) uses the term surface extension, for the process which is basically the ability to separate two marks along an axis. In the total hypocotyl the two marks are, the point of attachment of the coyledons, and the point of transition to the root. The increase in length over each 12 hourly interval is expressed as the absolute rate of surface extension in figure 3.2 and the relative rate of surface extension in figure 3.3. A comparison of the two rates shows that surface extension of the hypocotyl occurs in two phases. The first phase, from 0.5 to 1.5 days,

shows a low absolute rate of surface extension but a very high relative rate of surface extension. The second phase, from 2.5 days, conversely, is characterized by a high absolute rate but a minimum relative rate. Both rates reach a low level for a period between one and two days.

3.1.2 Hypocotyl Diameter

In transverse section, the shape of the hypocotyl is an ellipse with the wider diameter formed between the sides from which the cotyledons arise. The mean diameter of the hypocotyl at each age was determined as described in section 2.3. The mean diameter increased by a total of 1 mm between 0 and 3 days (figure 3.1 B). Figures 3.2 B and 3.3 B show the absolute and relative rates of increase in radius. The curves in this case are very similar in shape. The peak rate of increase in radius occurs at one day. This coincides with the time of very low rate of increase in hypocotyl length.

3.1.3 Hypocotyl Volume

The volume of the hypocotyl is a function of both its length and radius. The actual volume (figure 3.1 C) shows a smoother increase than either the length or radius because the radius increase occurs between the times of the first and second phases of hypocotyl surface extension.

The absolute volume increase shows a small peak at 1.5 days but the relative rate of volume increase shows a very high peak at this time. Following the pattern of hypocotyl length, there is a second peak of volume increase which is characterized by a high absolute growth rate and a lower relative growth rate.

3.1.4 Hypocotyl Weight

The mean wet weight and dry weight of one hypocotyl is shown in figure 3.4. The wet weight curve is similar to that for total hypocotyl volume (figure 3.1). The dry weight increases linearly between one and four days and is probably the result of synthesis of cell wall material from

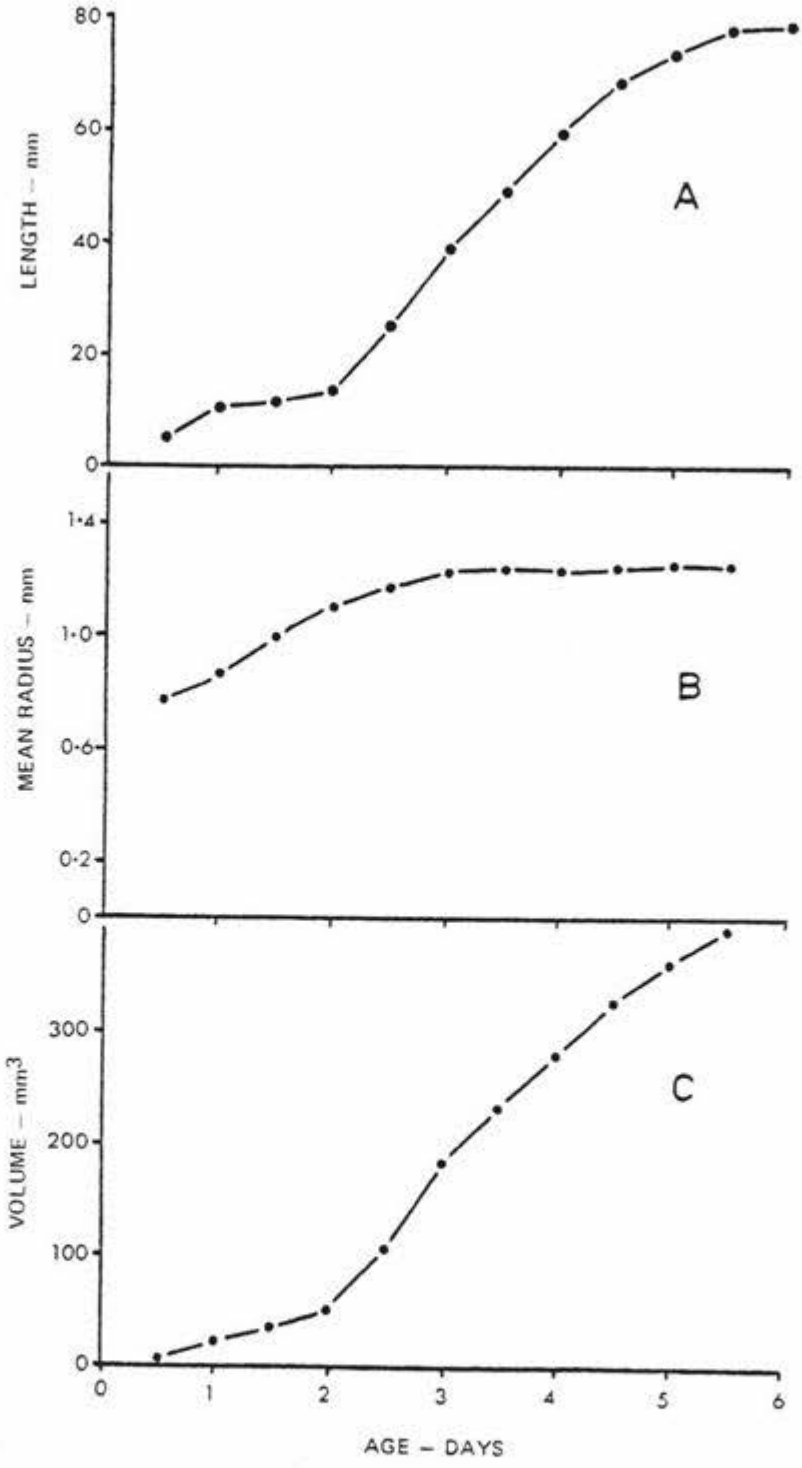


Figure 3.1 Dimensions of the Total Hypocotyl

A Hypocotyl Length

B Hypocotyl Radius: This is the mean radius calculated from the averages of the widest and narrowest diameters measured at 3 positions on the hypocotyls of 10 seedlings of each age. The S.E.M. for the mean of the diameters measured on 10 plants at each age and position was 2% or less of that mean.

C Hypocotyl Volume: This is calculated from the values in A and B.

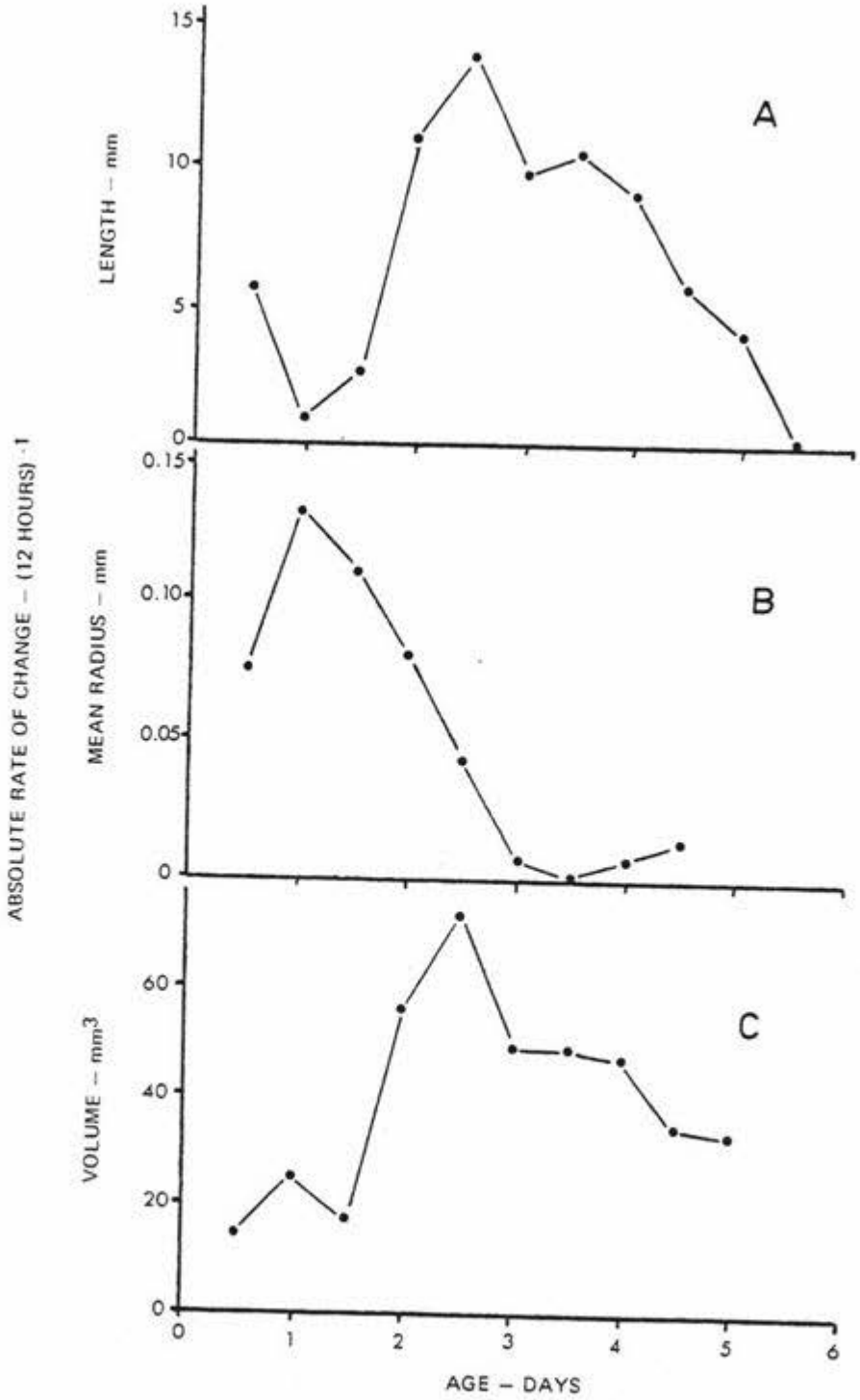


Figure 3.2 Velocity of Growth of the Hypocotyl

This is the absolute rate of change in dimensions.

A Length
B Mean Radius
C Volume

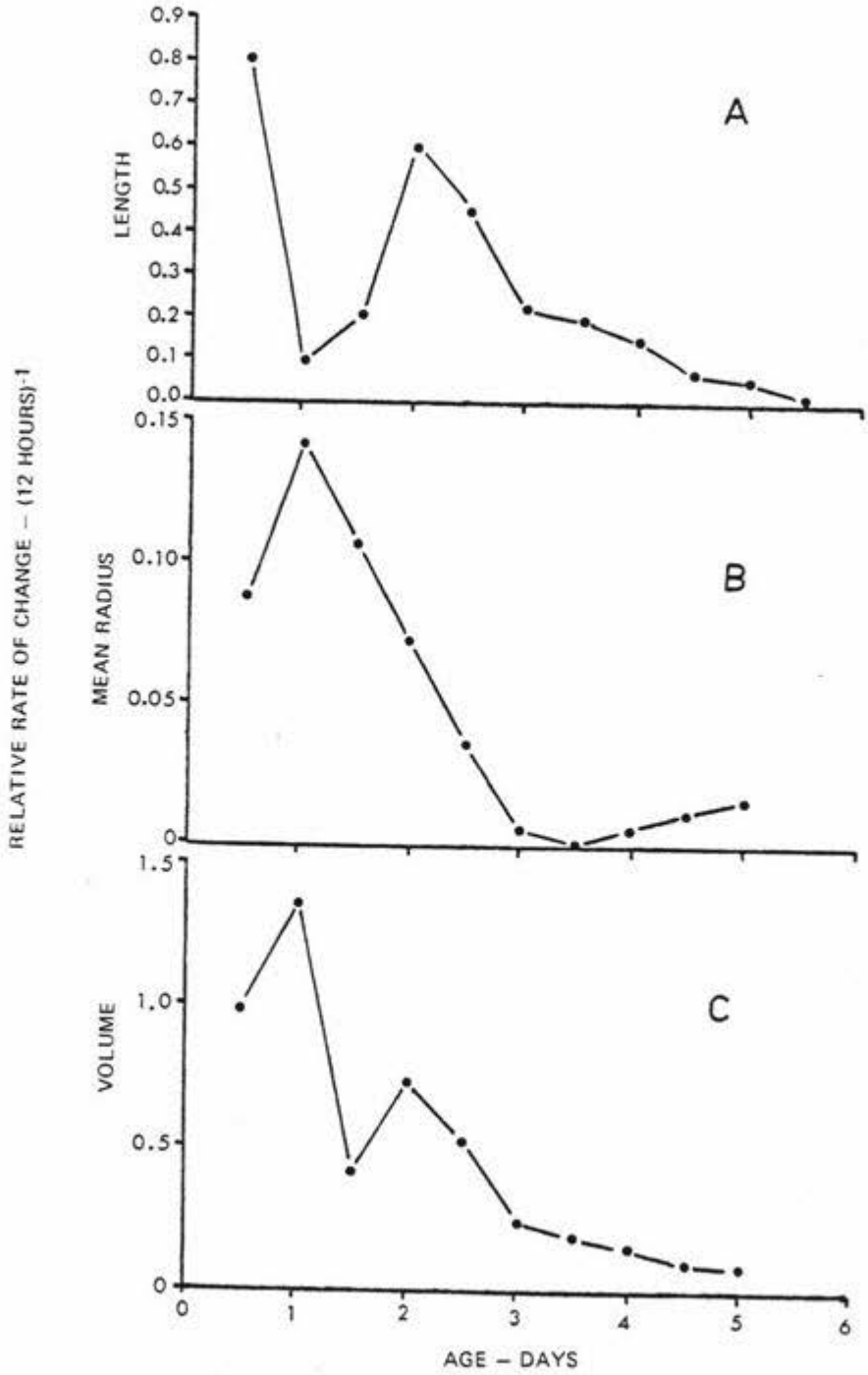


Figure 3.3 Relative Rates of Growth of the Hypocotyl

These are calculated using Green's formula for the relative rate of surface extension.

A Relative rate of longitudinal surface extension

B Relative rate of change of mean radius

C Relative rate of volume increase of the hypocotyl

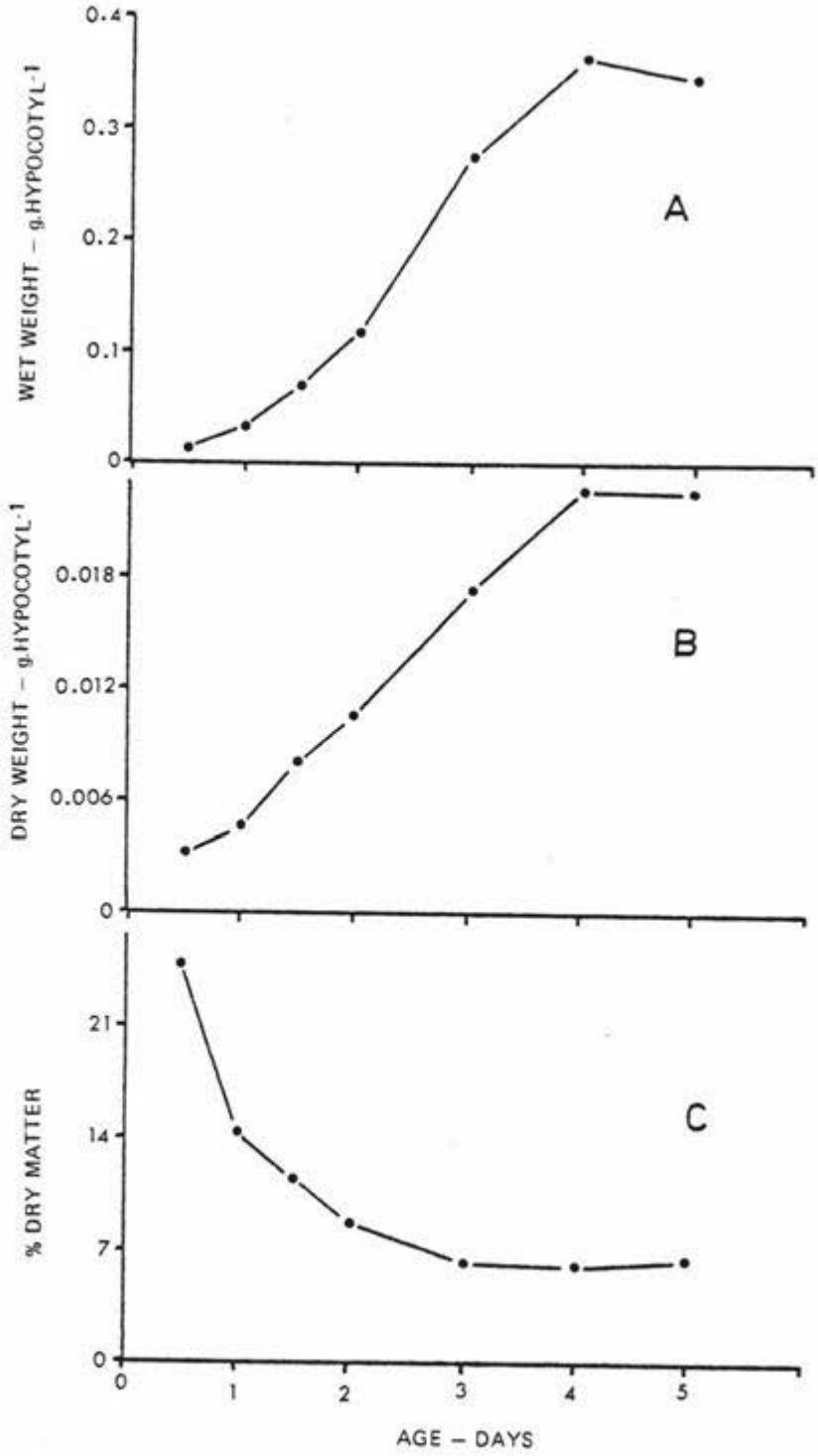


Figure 3.4 Hypocotyl Weight

A Mean wet weight of total hypocotyl (n=15)
B Mean dry weight of total hypocotyl (n=15)
C Percentage dry matter content of the hypocotyl: The hypocotyl of the dry seed has a dry matter content of 95%.

precursor material stored in the cotyledons.

The percentage of dry matter in the hypocotyl falls from 94% in the hypocotyl of the ungerminated seed to 24% after 12 hours, then falls more slowly to below 7% by 3 days (figure 3.4). The percentage dry weight was also determined for the upper regions 1 and 2 together, and for region 3. The upper regions had a dry matter content a few percent higher than for the total hypocotyl, and the dry matter content of region 3 was either the same or slightly below that for the whole hypocotyl.

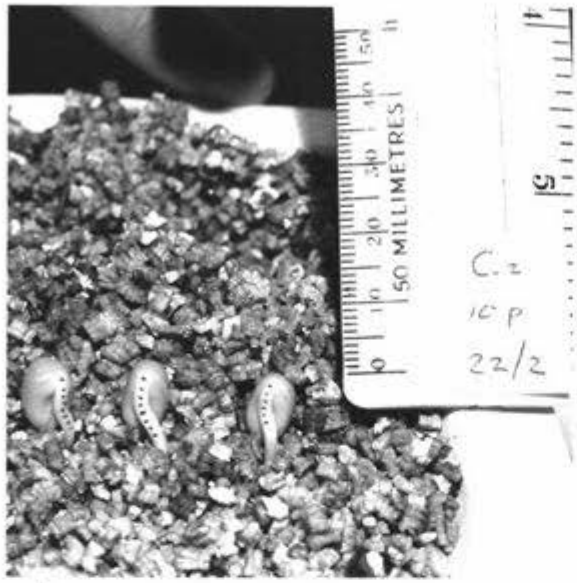
3.1.5 Distribution of Surface Extension in the Hypocotyl

Regions of elongation of the hypocotyl were determined by using the method of marking intervals on the hypocotyl and measuring the increase in length of each interval over a time period. This is the standard method for measuring surface extension, as described by Green (1976). The results are shown in figure 3.5. The reasons for the difference in total length of the hypocotyls of the two sets of plants at 3 days are firstly, that the hypocotyl is marked at 1 mm intervals from the top downwards and so a small region at the bottom is not included between two marks. This basal region elongates rapidly between 1 and 3 days, and this increase in length is not recorded. Secondly, the seedlings used from 1 to 3 days are exposed to more light than seedlings of that age would normally get under the standard growing conditions used. This is because they were uncovered for photography and between photography were covered over with only a thin layer of vermiculite. Under the standard growth conditions, seedlings do not break through the vermiculite until about 2 days so, in effect, are growing in the dark or very low light intensity until that age. Light strongly inhibits growth of the hypocotyl in *Lupinus albus* (Giles and Myers, 1964) and *L.angustifolius* (Van Oostveldt *et al*, 1976). So, the growth of seedlings marked at one day is inhibited by light from an earlier age than it is for

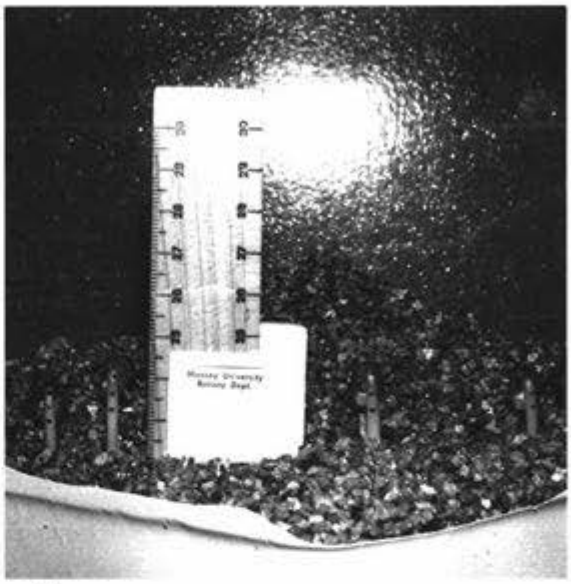
Plate 3.1

Monitoring Expansion of the Hypocotyl

A, B, C, and D are respectively 1, 2, 3 and 4 day old hypocotyls. Intervals on the 1 and 2 day old hypocotyls are marked with charcoal in lanolin paste, and on the 3 and 4 day old hypocotyls, with short sections of hair in lanolin paste. Seedlings were photographed at 4 hourly intervals and the change in distance between markers was measured from the photographic negatives.



A



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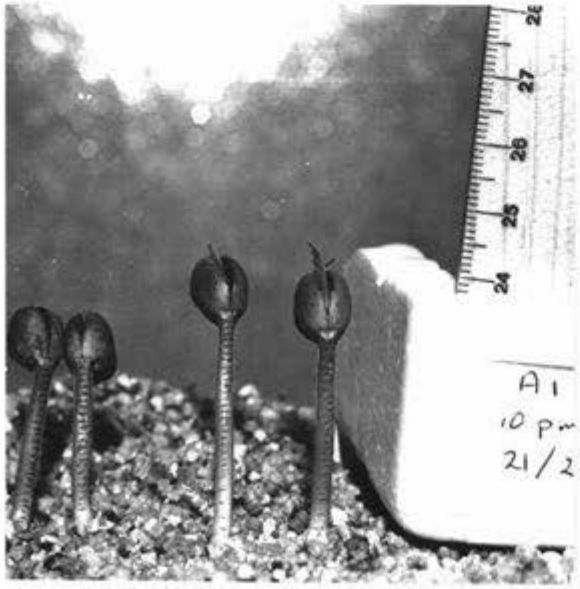
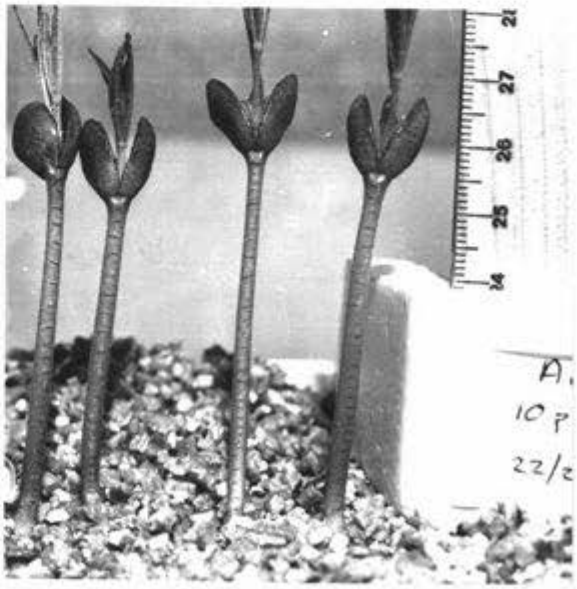


C



D

seedlings marked at 3 days.

For convenience, 3 regions of the hypocotyl were defined (see figure 3.5) and, for further study, tissue was taken from these regions. The relative rate of surface extension in the 3 regions, as calculated from data for figure 3.5, is shown in figure 3.6. A wave of rapid elongation proceeds from the base of the hypocotyl towards the top. Elongation ceases quickly in the basal region (3) after 3 days, and declines after 4 days in region 2. In region 1, elongation proceeds at a fairly steady rate over the whole expansion period and the rate does not reach as high a percentage as in the other two regions.

Figure 3.5 is drawn from the results of growth of eight seedlings in both the 1 to 3 day group and the 3 to 6 day group. There is a certain amount of variation in growth between different seedlings and an example is given in figure 3.7 of one particular seedling which elongated more rapidly and at an earlier age than the average. However, the pattern of growth is basically the same. In the basal region there is a high rate of surface extension which declines rapidly, and at the top is a fairly constant but low rate.

The pattern of expansion in the lupin hypocotyl is different from that in a typical growing axis. A root, for instance, has a meristematic tip which provides a constant supply of cells which elongate and push the tip in the direction of growth. Relative to the tip there is one constant region of elongation. The region of elongation in the lupin hypocotyl changes with age.

It is useful to know the stage of development of a region of tissue when it is used for the study of cytological features of its component cells.

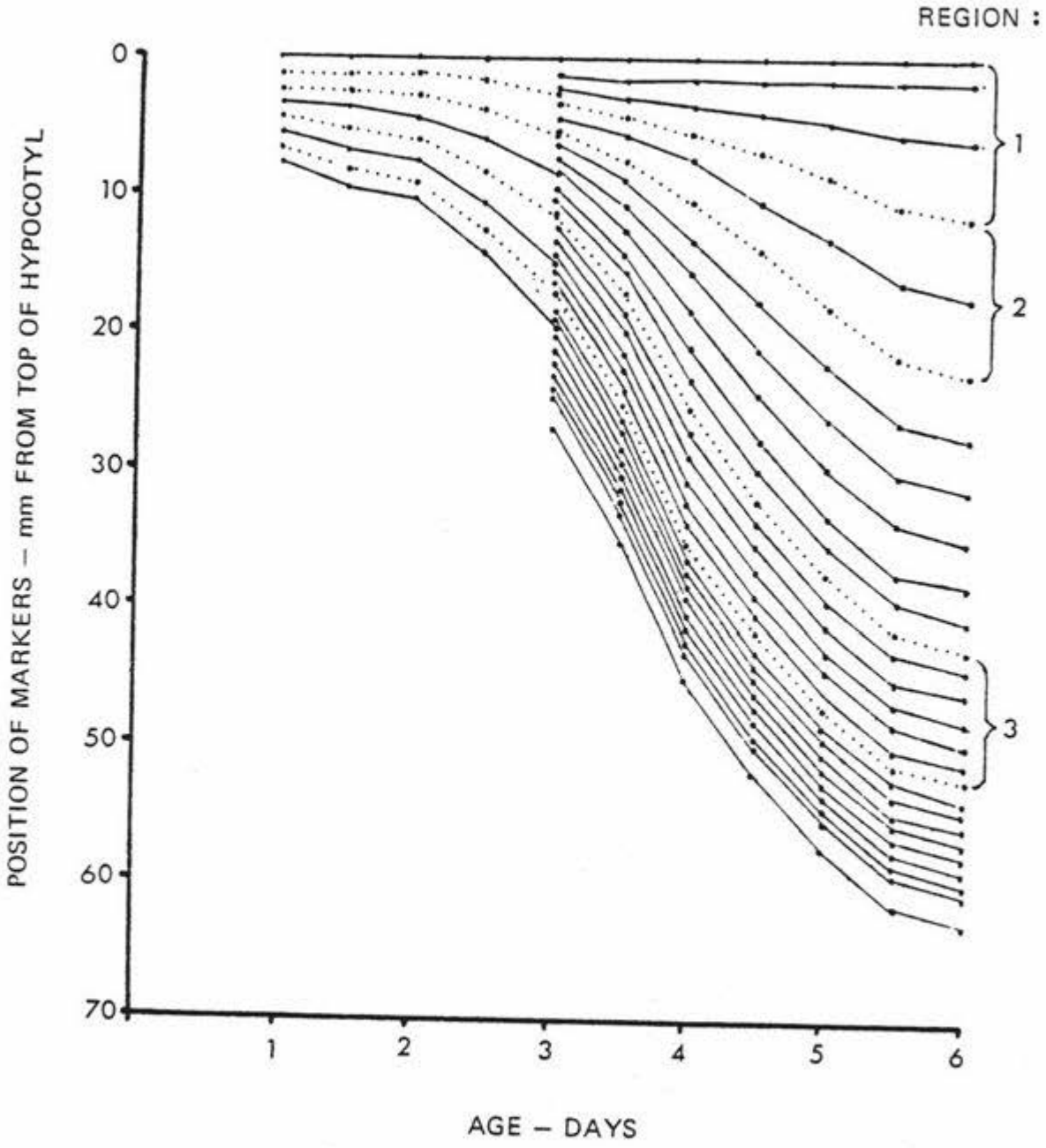


Figure 3.5 Distribution of Elongation in the Hypocotyl

Markers were placed 1 mm apart on the hypocotyls of one day old plants and on a new set of plants at 3 days old. The position of markers was measured as the distance from the top of the hypocotyl. The dotted lines mark the boundaries of the 3 regions from which tissue was selected for further studies.

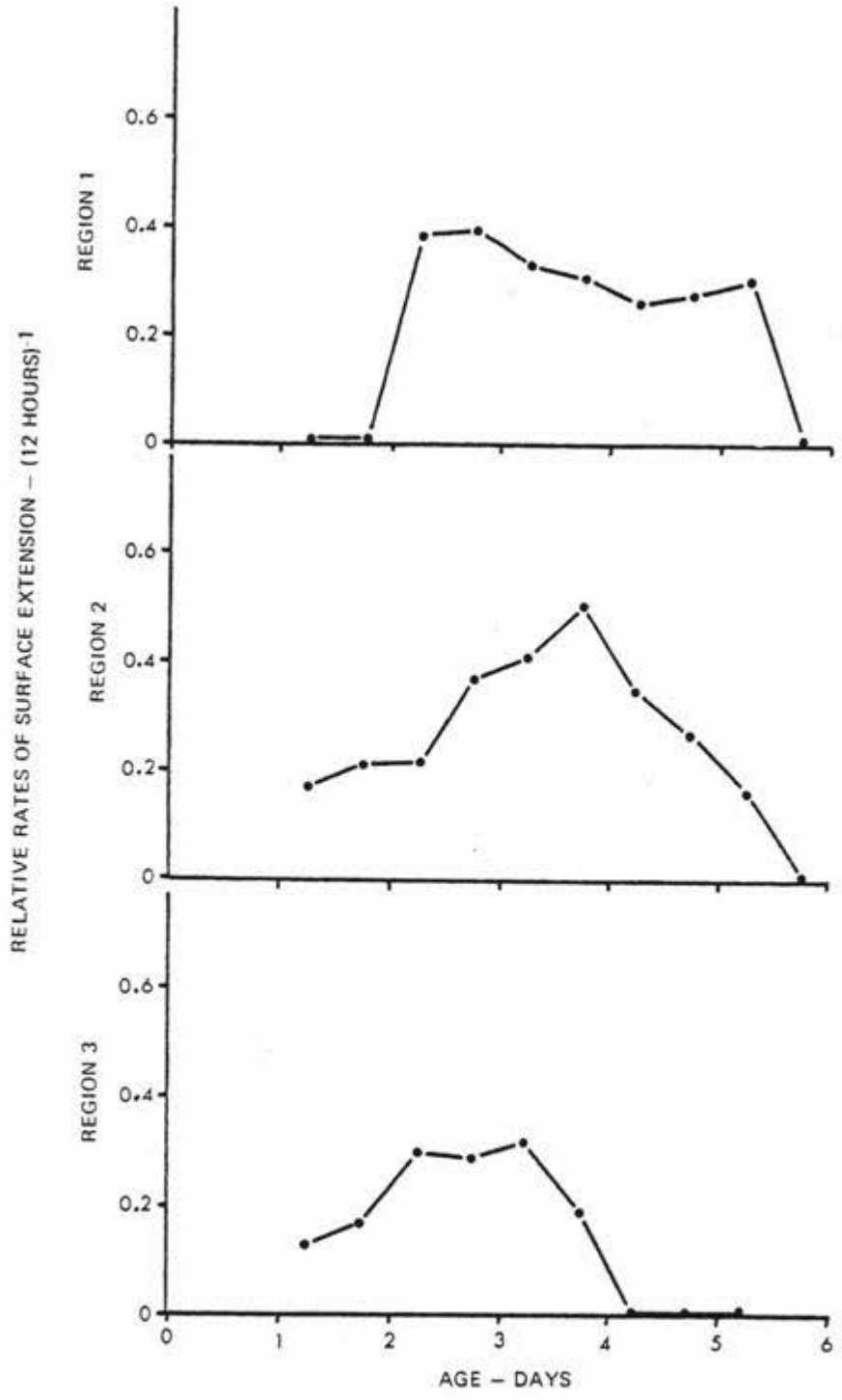


Figure 3.6 Relative Rates of Surface Extension in 3 Regions

These rates are calculated from data shown in figure 3.5.

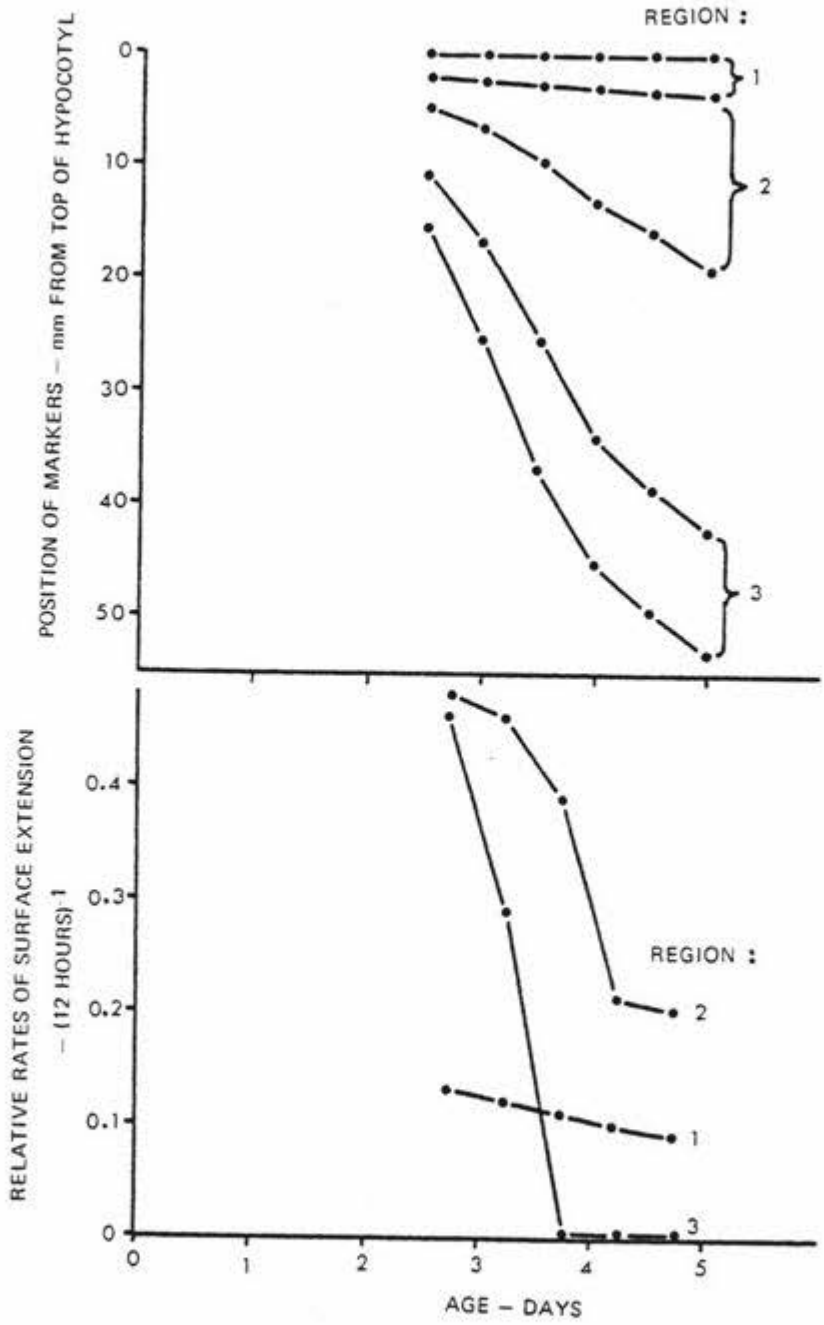


Figure 3.7 Elongation of an Individual Seedling

The three regions were marked on the hypocotyl at 2 days.
A The change in position of markers from 2.5 to 5 days
B The relative rates of surface extension in the three regions

3.2 Cell Dimensions

Cell length was measured on epidermal cells, outermost cortical cells and inner cortical cells in tissue from three regions of the hypocotyl (figure 3.8). The relative rate of increase in cell length for inner cortical cells is shown in figure 3.9.

The cells of the epidermis and outermost cortex are smaller than the inner cortex cells in both length and diameter (see figure 3.10). The volume of a 4 day epidermal cell (0.12 mm^3) is 28% of the volume of a 4 day inner cortex cell (0.45 mm^3).

Between one and two days the relative rate of increase in cell length is low, especially in the upper regions, 1 and 2. In region 2 the rate is actually negative, which means that the average cell length of inner cortex cells decreases over this time. It will be seen from section 3.3 that this is the time of peak mitotic activity, so a proportion of cells are halving their length. Epidermal cells do not show a decline in average cell length.

After 2 days the average cell length increases rapidly. Figure 3.9 shows that the relative rate of increase in cell length reaches a peak in region 3 first, between 2 and 3 days. Regions 1 and 2 show a peak between 3 and 4 days but the peak in region 2 is greater than in region 1. So it appears that cell elongation begins in the base of the hypocotyl and then proceeds acropetally to the top. The rate of cell elongation is slower at the top of the hypocotyl than in the lower regions. These results explain on the cellular level the distribution of surface extension of the hypocotyl, described in figure 3.5.

Surface extension begins at the base and moves up the hypocotyl, as does cell elongation. Region 1 has a fairly constant relative rate of surface extension and also a low peak rate of increase in cell length. By 4 days the relative rates of cell length increase and surface extension

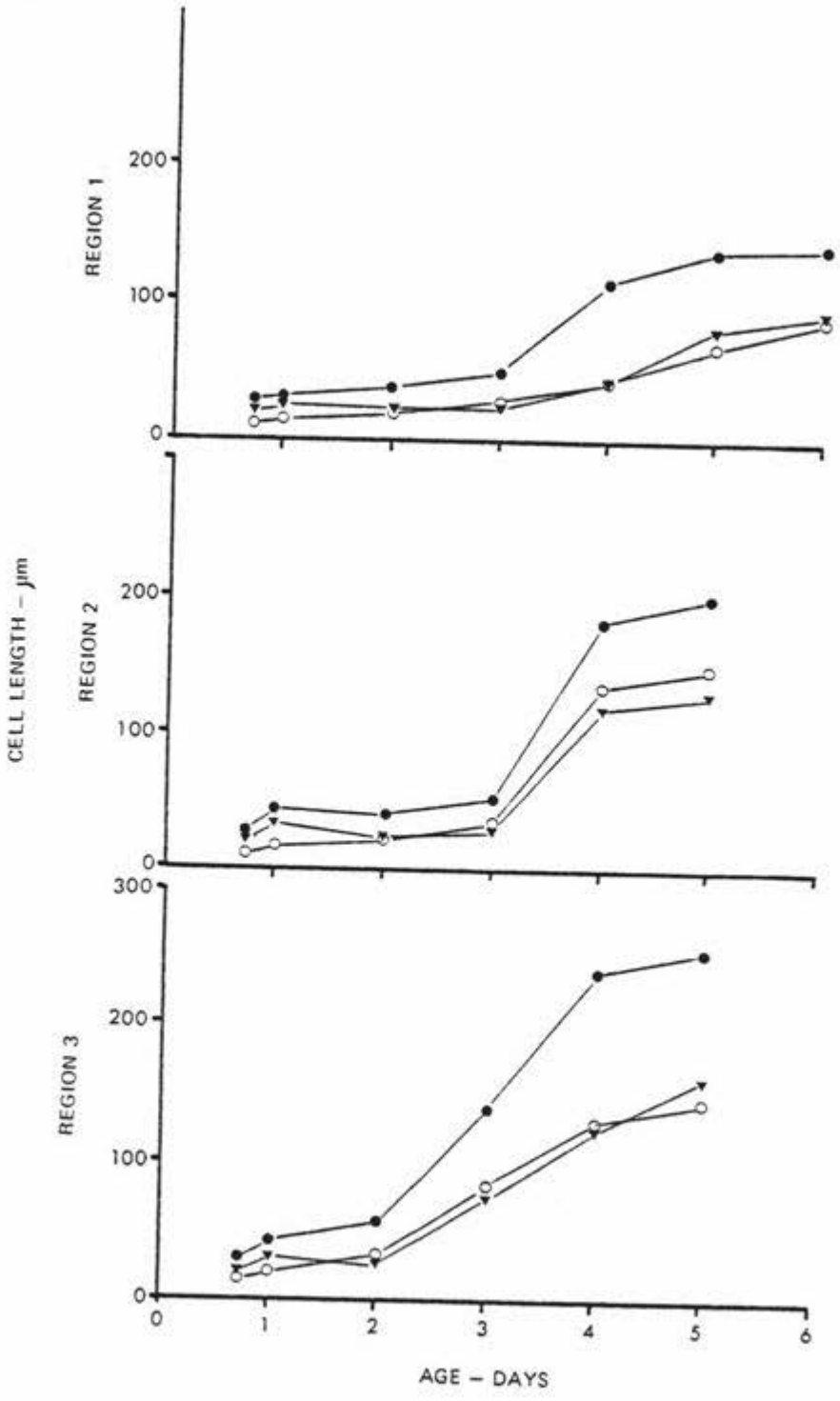
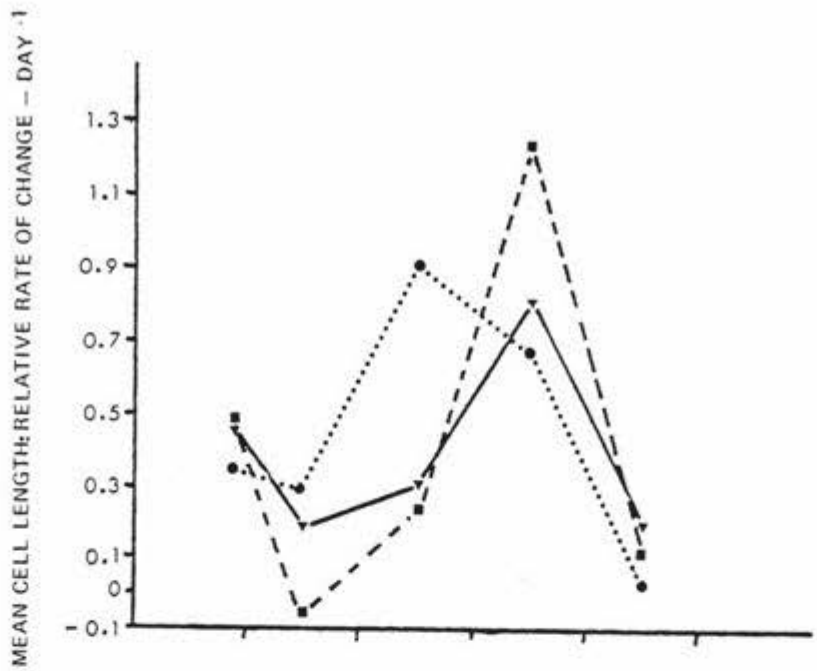


Figure 3.8 Cell Length

The cell types measured in each region were:
○ Epidermis
▼ Outermost layer of cortex
● Inner cortex

3.9



3.10

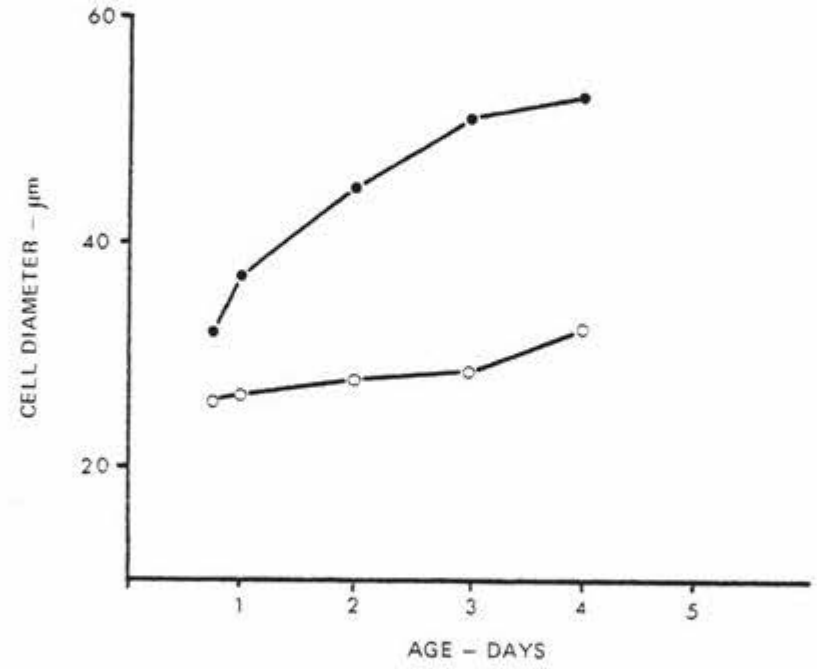


Figure 3.9 The relative rate of change in mean cell length for inner cortex cells from the three regions defined in figure 3.5

▼Region 1 ■Region 2 ●Region 3
The ratios are calculated by the formula $\frac{\ln L_1 - \ln L_0}{t}$, of Green (1976), from the data for inner cortex cells in figure 3.8.

Figure 3.10 Average Cell Diameter

● Inner Cortex ○ Epidermis
The diameter is the average of the means from each of the 3 regions. The average diameter for the outermost cortex layer was not significantly different from the average diameter of epidermal cells at any age.

are higher in region one than in the lower regions. Elongation continues in the top region of the hypocotyl after it has ceased in other regions. Cell elongation accounts for the surface extension pattern observed in the hypocotyl.

3.3 Cell Division

Cell division was estimated as the number of mitotic figures seen per 1000 cells observed. Estimates were made on vascular, cortical and epidermal cells in the three regions defined in figure 3.5. The results of cell division analysis are shown in figure 3.11.

Green (1976) introduces the term, cell partitioning for the act of completing a cross wall to form two new cells. This is an almost instantaneous point in the cell cycle and, in growth studies, it can be useful to define cell division in terms of a specific process which does not involve cell growth. The term cell division, may include cell growth which occurs in a meristematic region.

Cell division activity is first detectible in the vascular tissue of lupin hypocotyl at 1 day. There is a high frequency of mitotic figures in the basal region but only a low level in region 2. Mitosis is not detectible in region 1 until 1.5 days. This pattern is the same as for surface extension and cell elongation, where activity is initiated first in the basal region and moves acropetally.

Mitotic activity in the cortex is found at 1.5 days, when it occurs at its highest frequency in all regions. Cell division has ceased in the cortex of region 3 by 2 days, region 2 by 3 days, and region 1 by 4 days. Again the pattern is similar to that for other processes, with activity ceasing first in the basal region but continuing longer in regions 1 and 2. Region 1 is again characterized by a lower but more prolonged

level of activity than the other regions.

By 4 days, cell division in the vascular tissue has virtually ceased in region 3 but it continues at a low level in the upper regions of the hypocotyl.

Cell division was not detected at any stage in the epidermis.

The peak of cell division activity occurs at 1.5 days and it has reached a very low level by 3 days. This period of cell division coincides with the time when both total hypocotyl length and mean cell length are increasing at their lowest relative rates. Figure 3.3 A shows that there is a low relative rate of increase in total hypocotyl length between 1 and 2 days which is the same time that cell division activity is at a peak. Region 2 shows the highest peak of mitotic activity for cortex cells at 46 figures per 1000 nuclei, then region 1 with 26 per 1000, and region 3 with 23 per 1000. However, the regions are in the reverse order for magnitude of relative rate of cell length increase (figure 3.9) at 1.5 to 2 days. Region 3 has the highest rate, then region 1 , and then region 2 (whose cell division frequency is almost double that of regions 1 and 3) actually has a negative rate of cortical cell length change at this time. That is, the mean cell length is decreasing. Furthermore, cortical cell division ceases by two days in region 3, and 3 days in regions 1 and 2. Figure 3.9 shows that the peak rate of mean cell length increase occurs at 2.5 to 3 days in region 3, then 3.5 to 4 days in regions 1 and 2. That is, the maximum rate of mean cell length increase is apparent 0.5 day after the cessation of cortical cell division in all regions.

The growth pattern can also be described by comparison of surface extension and partitioning rates as defined by Green (1976). The partitioning rate cannot be calculated directly from the mitotic frequencies in figure 3.11 without knowing the lengths of both the cell cycle and the

mitotic phase. However, the frequency of mitotic figures would be proportional to the partitioning rate. Actual values for the partitioning rate can be calculated:

Relative Partitioning Rate = (Relative Extension Rate)
-(Relative Rate of Change in Mean Cell Length)

The values for the cortex tissue of region 2 are shown in figure 3.12 to show the relationship between the mean length of cells and the relative rates of surface extension and cell partitioning. When the surface extension rate is greater than the partitioning rate there is an increase in mean cell length, the size of the increase depending on the difference between the two rates. This is the situation most of the time in the example in figure 3.12. If the two rates are the same, the mean cell length is constant. When the partitioning rate exceeds the surface extension rate, then the mean cell length declines. This occurs for a short period (0.25 day) around 1.5 days in region 2 cortex tissue. The highest partitioning rate obtained in cortex tissue was 0.44 per 24 hours, i.e. 0.018 $hour^{-1}$. A peak rate of 0.15 $hour^{-1}$ was noted in the primary root of corn by Erickson and Sax (1956), (cited by Green, 1976), which is eight times faster than the maximum found for cortex tissue of the lupin hypocotyl.

The partitioning rate was calculated for epidermal cells over a 3 day period from 1 to 4 days, by the same method. The partitioning rate was found to be less than zero; that is, no cell partitioning occurred in the epidermis. During cell division analysis, no mitotic figures were observed in the epidermis. The implication is that cell elongation alone is sufficient to account for surface extension observed.

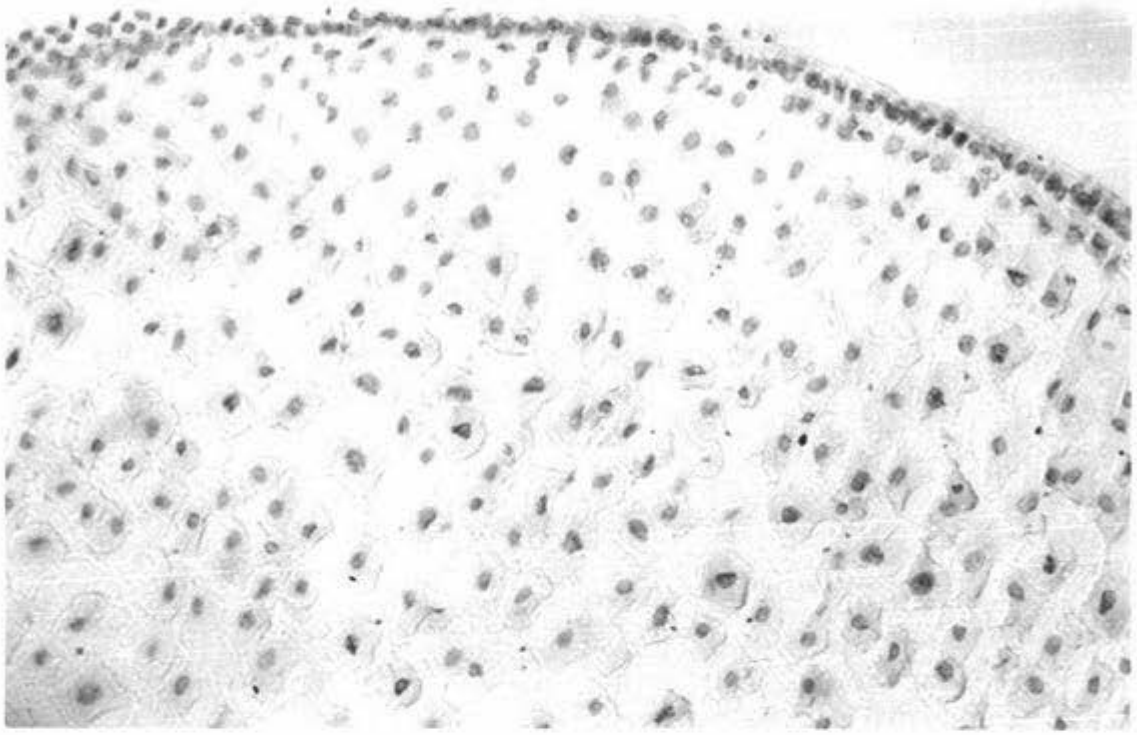
Cell division analysis was done on sectioned material, therefore the orientation of mitotic figures could be determined. The plane of division was perpendicular to the long axis of the hypocotyl in all mitotic figures in the cortex and most of the vascular tissue. Occasional mitotic figures

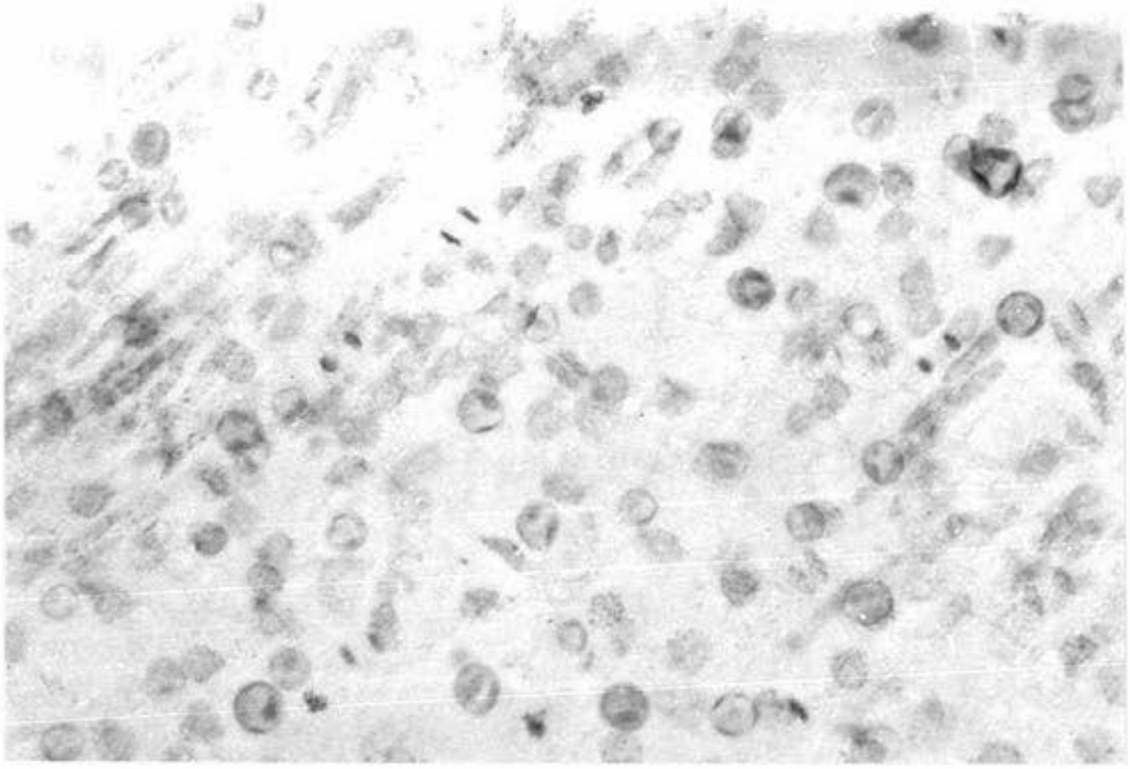
Plate 3.2

Cell Division Analysis

A. A 50 µm transverse section through the epidermis and cortex of a 1.5 day old hypocotyl. The tissue was stained by the Feulgen method and sectioned on a freezing microtome. This has advantages over the 'squash' technique as the precise location and the orientation of cell division can be known, and the layer of cells is of a constant thickness.
(X 200)

B. Longitudinal section of a region of cortex tissue near the vascular tissue (elongate nuclei are in vascular cells) in a 1.5 day hypocotyl. Various stages of cell division can be seen.
(X 500)





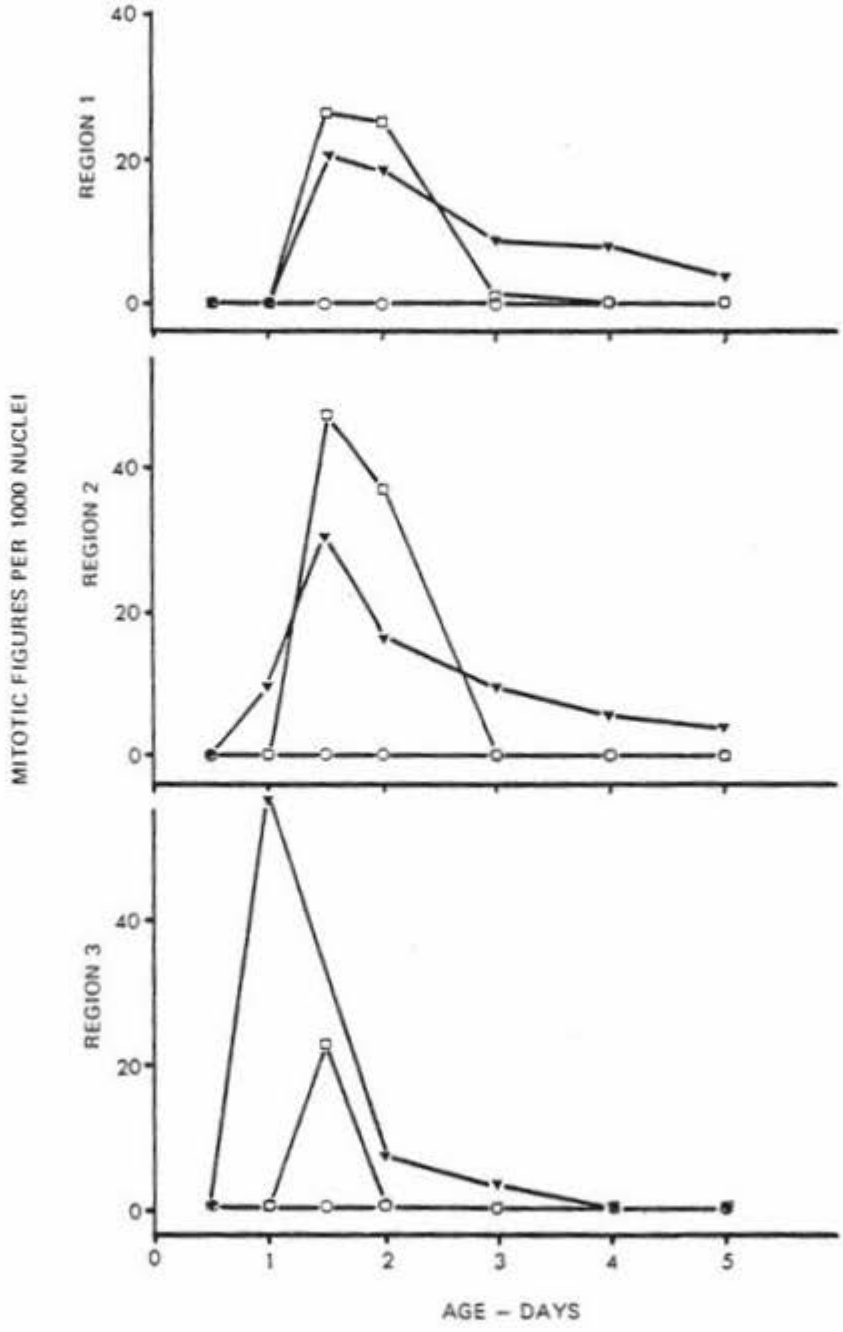


Figure 3.11 Cell Division

The frequency of cell division is expressed as the number of mitotic figures counted per 1000 nuclei.
○ Epidermis □ Cortex ▼ Vascular

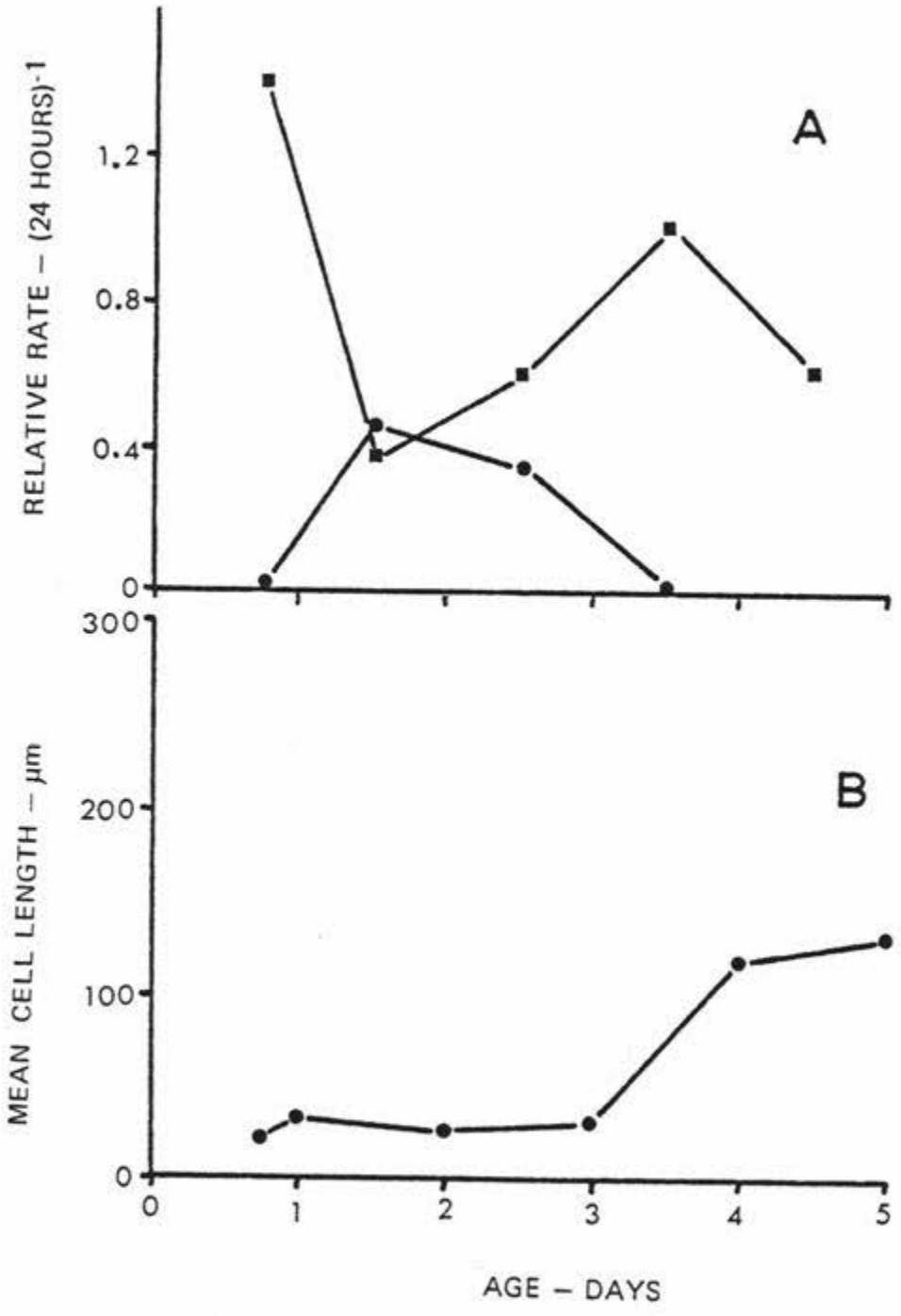


Figure 3.12 Analysis of Growth in Cortex Tissue of Region 2

A Relative rates of surface extension (■) and cell partitioning (●) per 24 hours
B Mean length of cortex cells in region 2 (from figure 3.7)

in the vascular tissue were orientated parallel to the long axis of the hypocotyl.

3.4 Conclusions

The hypocotyl seems to be well formed during seed development. Following soaking seeds in water, there is an initial period of rapid elongation from 0.5 to 1.5 days, as shown by measurement of the total hypocotyl length. From the study of cell lengths in the upper and lower regions of the hypocotyl between 16 hours and 1 day it appears that this initial expansion occurs over the whole length of the hypocotyl. This burst of elongation pushes the radicle and base of the hypocotyl out through the testa into direct contact with the surrounding medium.

After this period of rapid elongation there is a period of 1 to 2 days during which time average cell length either increases only slowly or in some cases decreases slightly. This period coincides with the time when cell division occurs, between 1 and 3 days and, by halving the length of at least some cells, accounts for the decrease in average cell length found in cortical tissue.

Following the division phase, total hypocotyl length increases rapidly from 2 to 4 days. The relative rate of surface extension slows down after 4 days and has virtually ceased by six days. The distribution of regions of elongation within the hypocotyl changes during this expansion phase, with a wave of elongation proceeding from the base to the top of the hypocotyl. The period of elongation is brief at the base and becomes longer towards the top. This pattern is shown both by surface extension of marked hypocotyls and cell length analysis.

The cell elongation and cell division processes start at the base of the hypocotyl and proceed to the top. This suggests that these processes

may receive some stimulus from the root.

The interaction of the developmental processes of cell division and cell elongation in the pattern described leads to the morphological changes which are observed during the growth of the lupin hypocotyl.

CHAPTER FOUR CYTOLOGICAL CHANGES DURING CELL DEVELOPMENT

In the previous chapter the growth of the hypocotyl was described in terms of the regions of cell division and cell elongation. In this section some of the cytological changes associated with these developmental processes are described.

4.1 The Nucleus

4.1.1 Nuclear Diameter

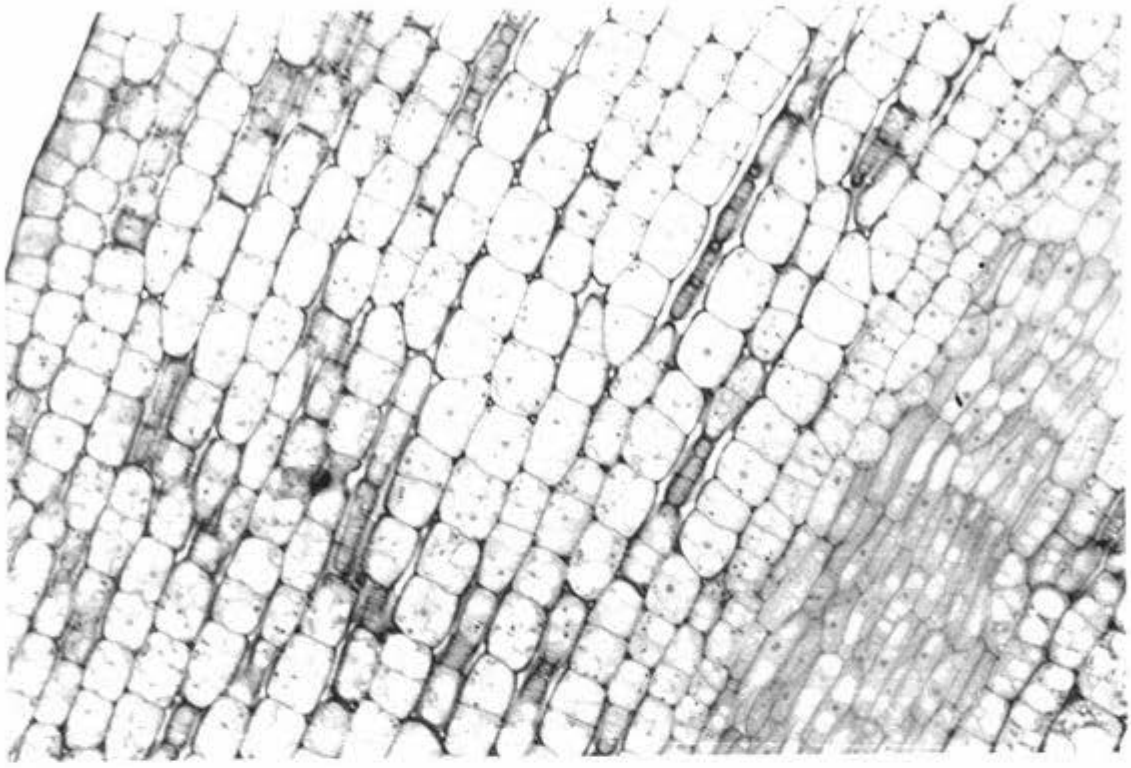
The shape and position of the nucleus within a cell varies with the cell's stage of development. Before one day, cells generally have a centrally located nucleus which is approximately spherical in shape. Plate 4.1 shows that from an early stage cells have a large central vacuole, so the nucleus is usually located against the cell wall. Plate 4.2 shows an elongating cell where the nucleus is still located in the centre. Its shape is irregular and varies between hemispherical and oblate. In the older tissue prepared for light microscope examination, there was a low frequency of cells in which nuclei were seen. The nucleus is smaller and each cell is much larger so there is a lower probability of nuclei being included in a section of a certain area. The nucleus was found to range in size from 8 to 12 μm in diameter. The mean diameter for nuclei of one day cortical tissue was 10.2 μm and in five day tissue was 9.5 μm. This 7% decrease in diameter from one to five days may occur as a result of a decrease in nucleolar size over this time.

The maximum and most usual length for an anaphase figure during mitosis was 11 μm (plate 3.2). The lupin hypocotyl nucleus is small in comparison with that of root meristem cells of *Vicia faba*, where the nuclear diameter averages 14.8 μm and the usual length of anaphase figures is 25 μm.

Plate 4.1

A. Longitudinal section of hypocotyl tissue from region 2 of a one day old seedling. This tissue was prepared for measurement of cell dimensions as described in section 2.5. Cells are already well vacuolated.
(X 170)

B. Transverse section of tissue shown above. Many small vacuoles join to form the large central vacuole. As the vacuole forms, the nucleus is usually located at the periphery of the cell. The nucleolus is consistently spherical but the nucleus has a varied outline. A central nucleolar vacuole (nv) can be seen within two nucleoli.
(X 1300)



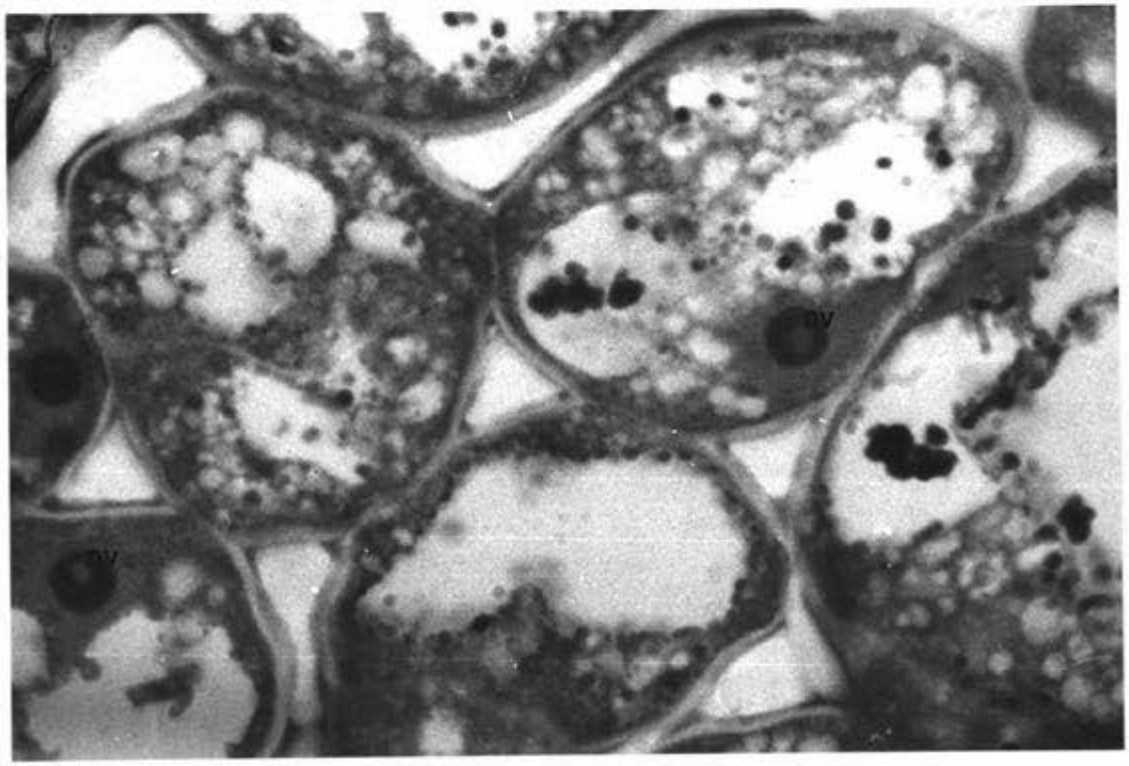
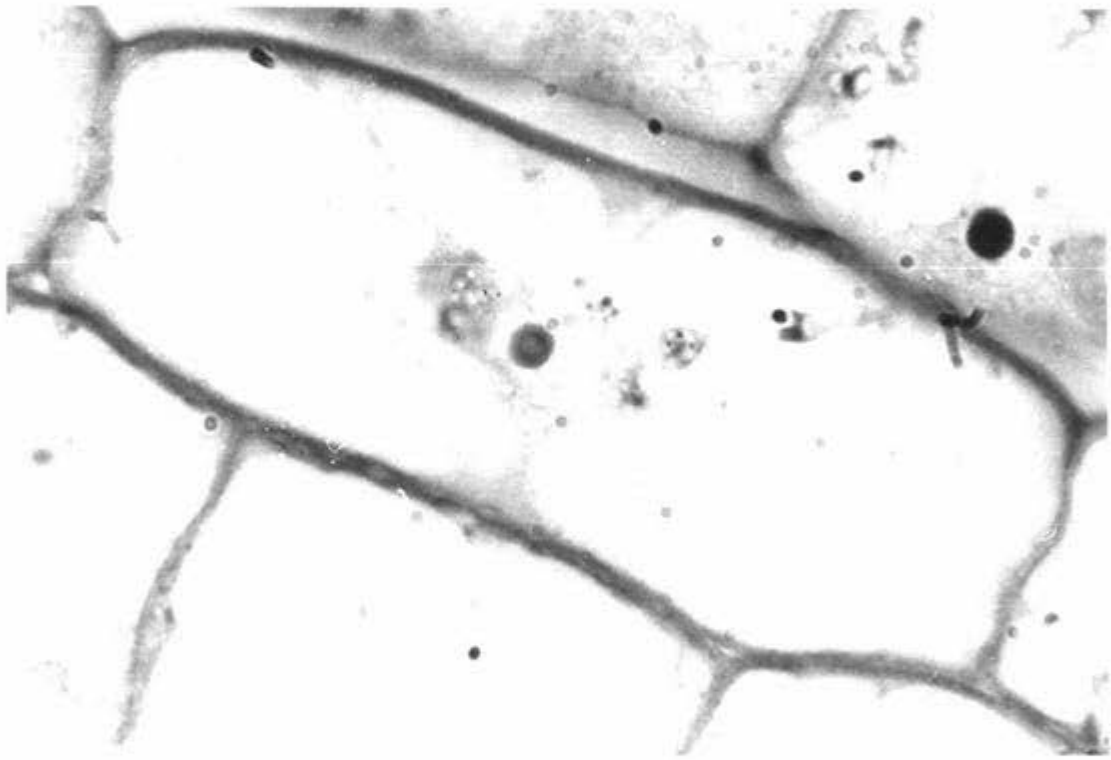

PV
PV

Plate 4.2

An elongating cortex cell in region 2 of a 2 day old hypocotyl. In this case the nucleus has remained in a central position in the cell. It is held in that position by strands of cytoplasm. The nucleoli in this tissue are smaller and look more compact than nucleoli of cells at an earlier stage of development.

(X 1300)



The measurements on Vicia were made on tissue prepared by the Feulgen method for cell division analysis, the same as was used for lupin hypocotyl tissue.

4.1.2 Nucleoplasm

From electron micrographs, the chromatin in the nucleus appeared to be mostly dispersed (plate 4.3 A). Small regions of the condensed chromatin were often seen to be associated with the nuclear membrane (plate 4.4 and others).

Perichromatin granules were frequently seen in the nucleoplasm, particularly in nuclei of cells at an early stage of development. They are densely-staining particles, variable in shape and ranging in size from 200 to 400 Å diameter (plate 4.14). Chaly and Setterfield (1975) suggest that the smaller granules represent ribosomal precursors in transit. However, in general, the function of perichromatin granules is not yet established.

4.1.3 Nuclear Membrane

The nuclear membrane shows varying degrees of convolution (plate 4.3 B). In tissue up to 3 days old, the nuclear membrane may be very folded and the nucleus may contain cytoplasmic inclusions as shown in plate 4.5 A. This gives a large surface area for interaction between the nucleus and cytoplasm. In plate 4.5 A, pores are seen in the nuclear membrane. They were seen commonly during the first two days of hypocotyl growth. Pores were found to measure 850 Å in diameter. In face view they appear as an electron-dense ring of material enclosing a core of material of equal or greater electron density (see plate 4.5 B). The frequency of pores in the nuclear membrane was estimated from nuclei of 1 and 2 day old tissue. After 3 days, nuclei were found infrequently in cortex cells, and in those found, nuclear pores were either absent or not easily distinguishable. The mean frequency of pores in one day old tissue was 51.7 pores in an area of nuclear membrane

measuring one square micrometer. In the two day tissue the pore frequency was 34.9 pores per square micrometer of nuclear membrane. Calculation shows that a nucleus with diameter 10 μm, as is found in one day tissue, may have a total of 17,000 pores in its nuclear membrane. This number of pores would allow for controlled transfer of a considerable amount of material between the nucleus and cytoplasm.

Ribosomes are sometimes seen, as in plate 4.4 A, very close to the outer surface of the nuclear membrane. It is not known if these ribosomes are actually associated with the membrane or if they form any pattern on the surface.

The endomembrane concept of Morré and Mollenhauer (1974) suggests that endoplasmic reticulum is formed from nuclear membrane. If this is so, it may be that ribosomes are already attached in a particular pattern when membrane buds off to form rough endoplasmic reticuli. Possibly in some cases, the outer nuclear membrane is a substrate on which ribosomes are arranged in a particular configuration before becoming functional polysomes on rough endoplasmic reticuli.

4.1.4 Karyosomes

A spherical body was occasionally seen associated with the nucleolus (plate 4.4 A). It was found to measure about 0.6 μm in diameter, and appeared fibrillar in nature, but was slightly less electron-dense than the fibrillar region of the nucleolus. Possibly it occurs often in the nucleus but, because of its small size, it infrequently appears in ultrathin sections. They fit the description of karyosomes given by Chaly and Setterfield (1975). At present their functional significance has not been determined.

4.1.5 Nucleolus

The nucleolus is a prominent feature of the nucleus because of its large size and high electron-density when prepared for electron microscopy.

The size of nucleoli was measured on tissue prepared for measuring cell length (section 2.5), as shown in plate 4.1. The diameter ranged from 2 to 7 μm depending on cell type and age of the hypocotyl. Table 4.1 gives the nucleolar dimensions at the ages and positions where they were accurately determined.

Table 4.1 Diameter of Nucleoli from Epidermal and Cortical Cells (The S.E.M. is given as a percentage of the mean.)

Hypocotyl Age (days)	Region	Nuclear Diameter (μm) Epidermis	Cortex
0.6	1	3.73 ± 7%	4.36 ± 3%
1.0	1	3.36 ± 8%	4.52 ± 5%
1.0	3	3.27	4.60 ± 6%
2.0	1	3.12 ± 3%	3.40 ± 3%
2.0	3	3.07 ± 5%	3.54 ± 9%
3.0	1	2.31 ± 2%	2.58 ± 7%

Nuclei were seldom found in 4 day cortex tissue as the cells were very large and had an extremely thin layer of cytoplasm around the periphery. The trend that these figures show is that nucleoli reach their maximum diameter within the first day of germination. The diameter declines by between 30 and 40 percent over the following 2 days. In the epidermis the volume of the nucleolus declines from 10.7 μm^3 to 4.1 μm^3 which is a reduction to 38% of its volume at 1 day. The change in nucleolar volume in cortical cells is from 15.9 μm^3 at 1 day to 5.3 μm^3 at 3 days, which is a reduction to 33% of its volume at 1 day.

The ultrastructure of 16 hour and 3 day nucleoli was compared and it was found that the 60% reduction in volume is accounted for by condensation of vacuolar regions and loss of granular regions.

Plate 4.9 A shows the nucleolus of a cortical cell in 16 hour tissue.

It has a diameter of 4.4 μm. In this nucleolus and those seen under the light microscope (plate 4.1 B) vacuoles can be seen. Several nucleoli in plate 4.1 B contain one large central vacuole.

Chaly and Setterfield (1975) distinguish between two types of vacuoles; large storage vacuoles often containing some granular material, and smaller ones, referred to as lacunae, found in the fibrillar regions. Lacunae contain dispersed fibrillar material and a small amount of granular material. In plate 4.6 B, dispersed fibrillar material is marked. The expanded DNA template, upon which ribosomal material is formed, may be what appears as the small lacunae. Possibly, as the ribosomal material forms, it accumulates in a large vacuole before being expelled into the nucleoplasm. Not many sections showed nucleolar vacuoles opening into the nucleoplasm but they were occasionally seen, as shown in plate 4.6 A. In the light microscope study these openings were seen more frequently because sections were much thicker (5 μm) and one could focus at several planes through the nucleolus.

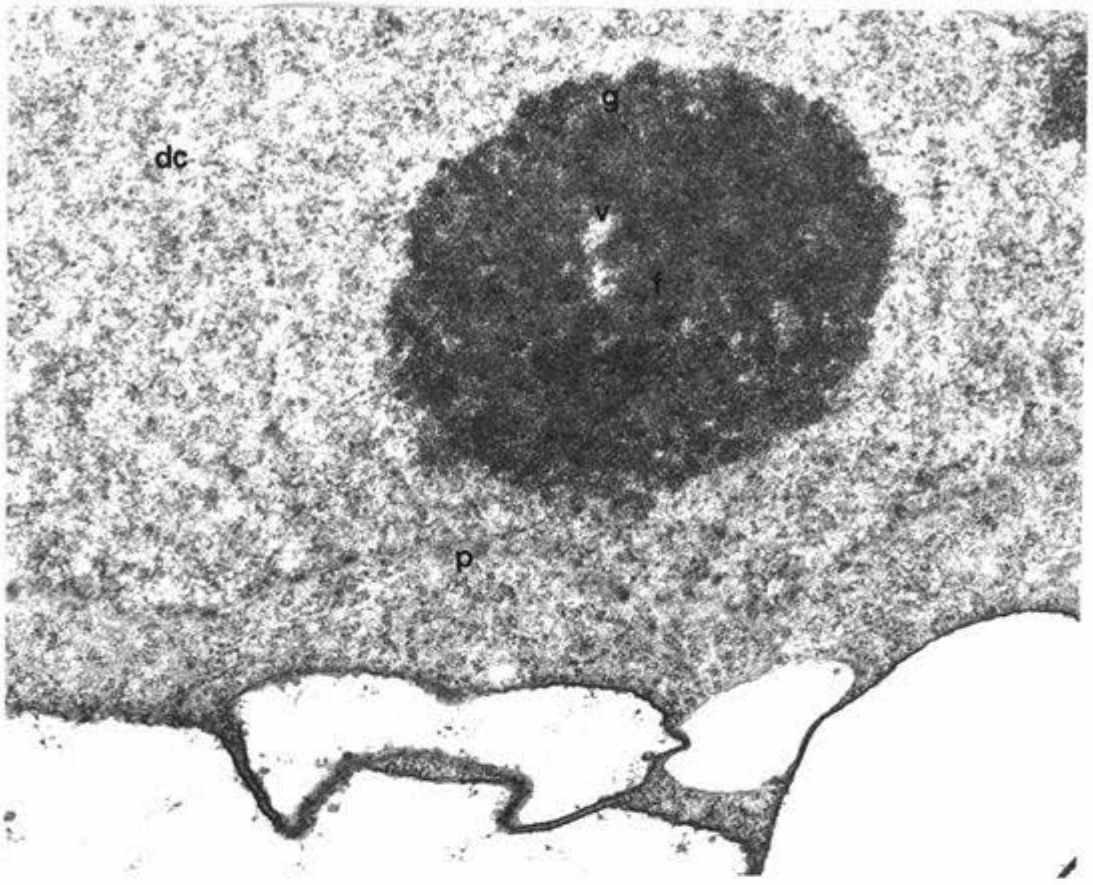
The nucleoli of cells from tissue up to 2 days old contained a large proportion of granular material which formed zones at the periphery of the nucleolus (see plates 4.5 A and 4.6). Possibly these granules are formed on the outside of the nucleolus rather than internally. There would seem to be some advantage to the nucleolus in maintaining a spherical shape. The fibrillar portion does not always form a sphere, but the granular zones fill it out to a spherical shape. The spherical shape would not be maintained if there was a random drift of granular material into the nucleoplasm.

By 3 days the nucleoli of most cells have shrunk in size and, in the light microscope preparations, are more densely staining than those at one day (plate 4.2). The extent of vacuoles is greatly reduced and the granular zone is narrow and peripheral.

Plate 4.3

A. Nucleus of an epidermal cell in the top region of a 1 day old hypocotyl, showing dispersed chromatin (dc), numerous pores (p) in the nuclear membrane, nucleolus with granular (g), fibrillar (f) and vacuolar (v) zones.
(X 22,500)

B. Longitudinal section of cortex cell in region 3 of a 1 day old hypocotyl. The nuclear membrane is convoluted, increasing the surface area for interaction between the nucleus and cytoplasm. There is a region of condensed chromatin (cc) adjacent to the nuclear membrane.
(X 17,500)


g
dc
v
f
p

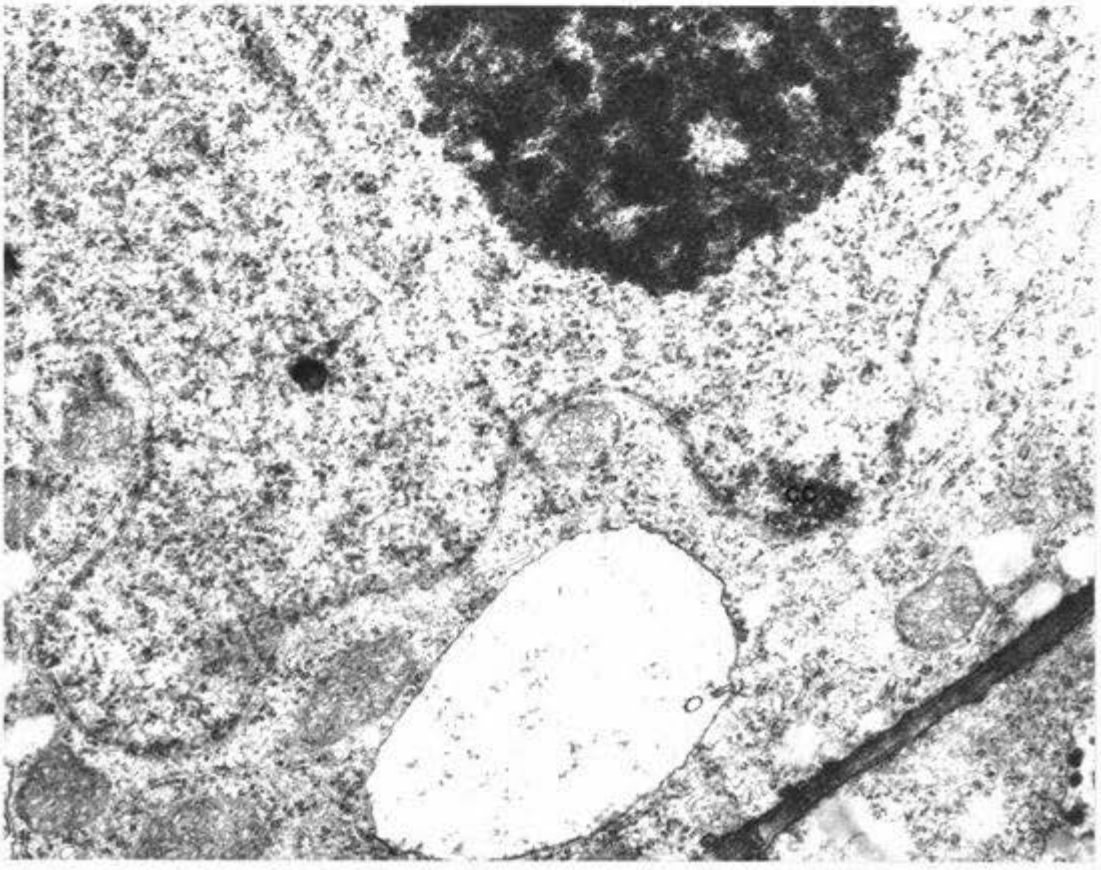
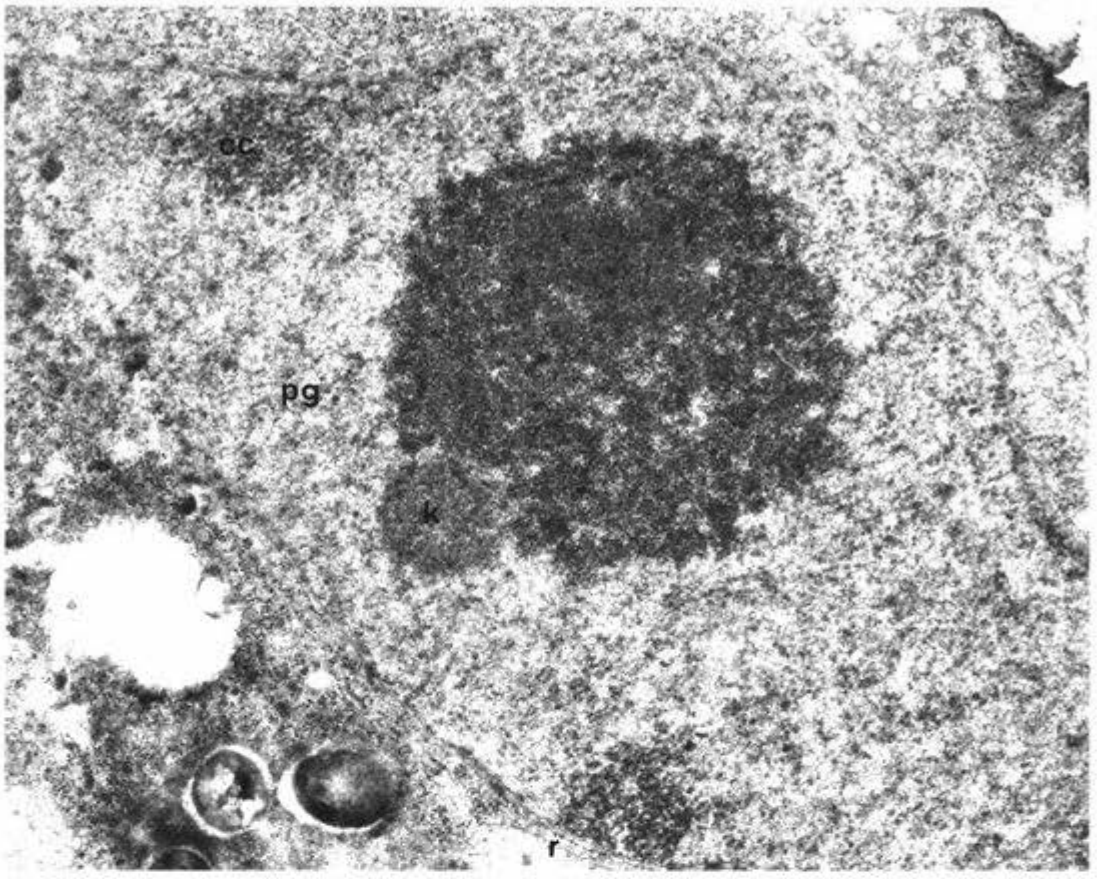

cc

Plate 4.4

A. Transverse section of an epidermal cell in the top region of a one day old hypocotyl.
Associated with the nucleolus is a karyosome (k) which appears to be fibrillar, although not as densely staining as the fibrillar region of the nucleolus. Regions of condensed chromatin (cc) occur within the nucleoplasm. Some ribosomes (r) are found adjacent to the nuclear membrane but it is not known how close the association is. Within the nucleoplasm are some densely staining perichromatin granules (pg).
(X 22,500)

B. Condensed chromatin (cc) located adjacent to the nuclear membrane in a cortex cell from region 2 of a 2 day old hypocotyl.
(X 74,000)


cc
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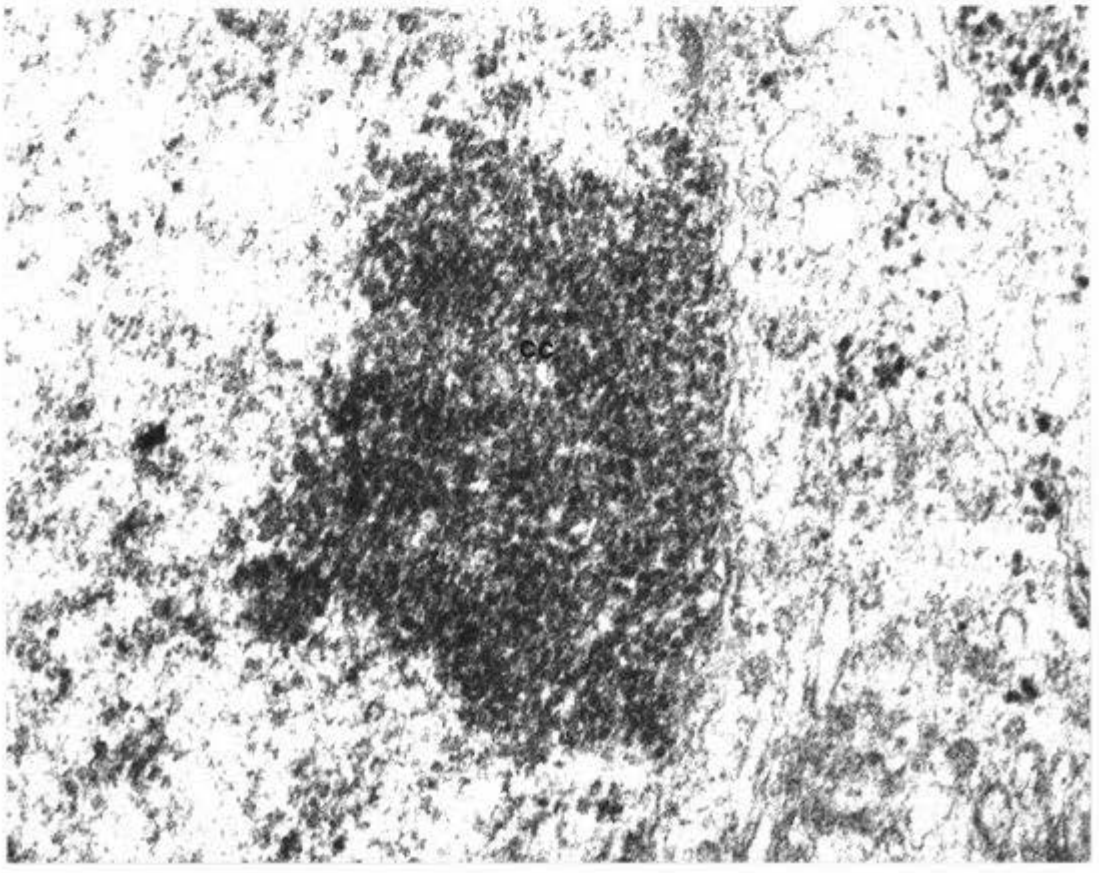
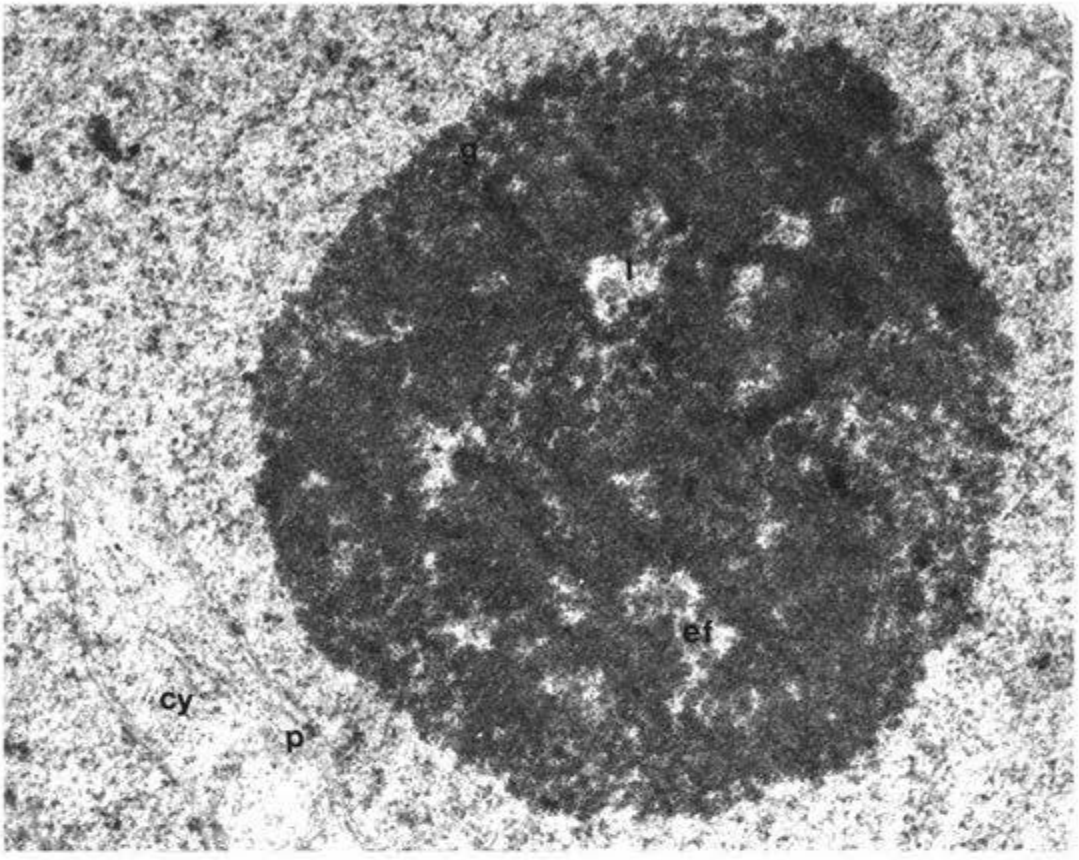

cc

Plate 4.5

A. The nucleolus of an epidermal cell in region 3 of a 1 day old hypocotyl. It has a diameter of 4 µm which is one of the largest measured in the epidermis during this study. It has extensive granular regions (g). Some of the lacunae (l) contain a slightly less densely-staining fibrillar material which may be regions of nucleolar DNA which have expanded in order to function as the template on which ribosomal RNA is formed. Plate 4.6 B shows a part of this nucleolus at a higher magnification. ef = expanded fibrillar material. This section shows a projection of cytoplasm (cy) into the nucleus and some pores (p) are present.
(X 22,500)

B. A tangential section through the nuclear membrane in an epidermal cell in the top region of a 2 day old hypocotyl shows nuclear pores (p) in face view. They have an outer ring of electron-dense material enclosing a core of material of equal or greater electron density. The pores measure about 850 Å in diameter. n = nucleoplasm, cy = cytoplasm
(X 99,000)


cy
p
ef

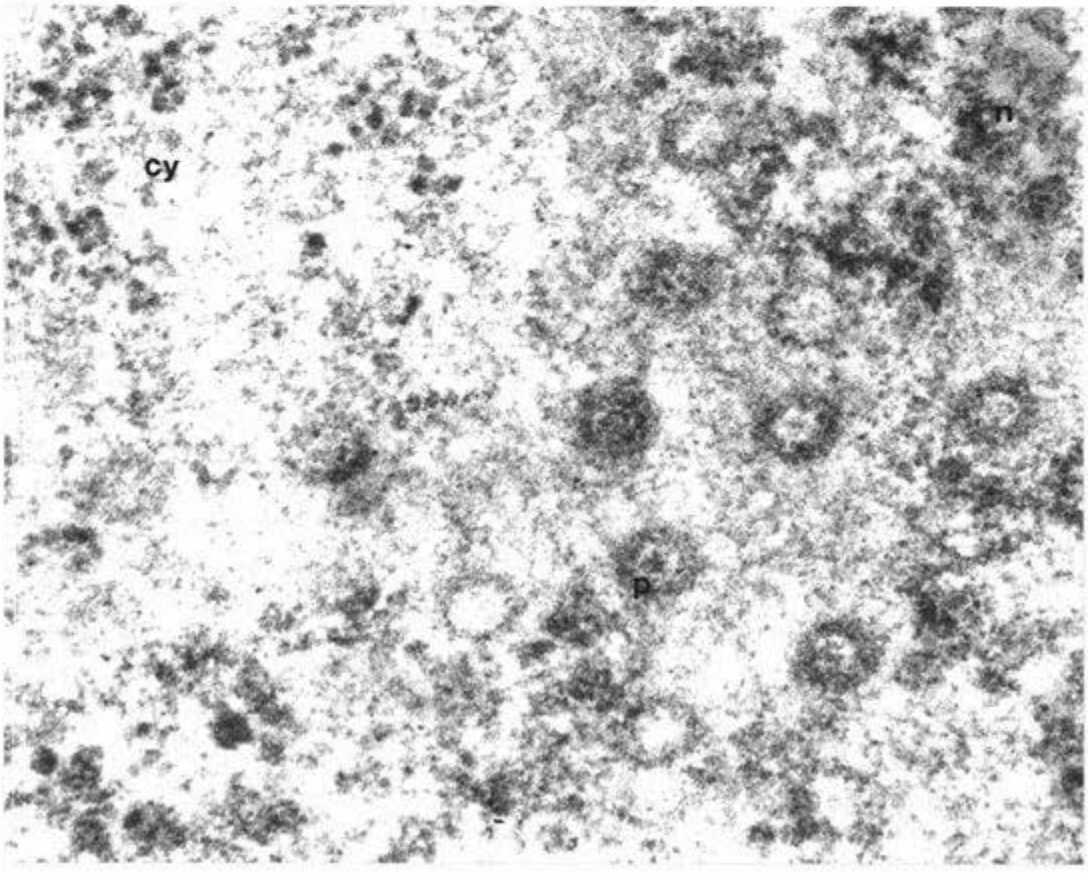
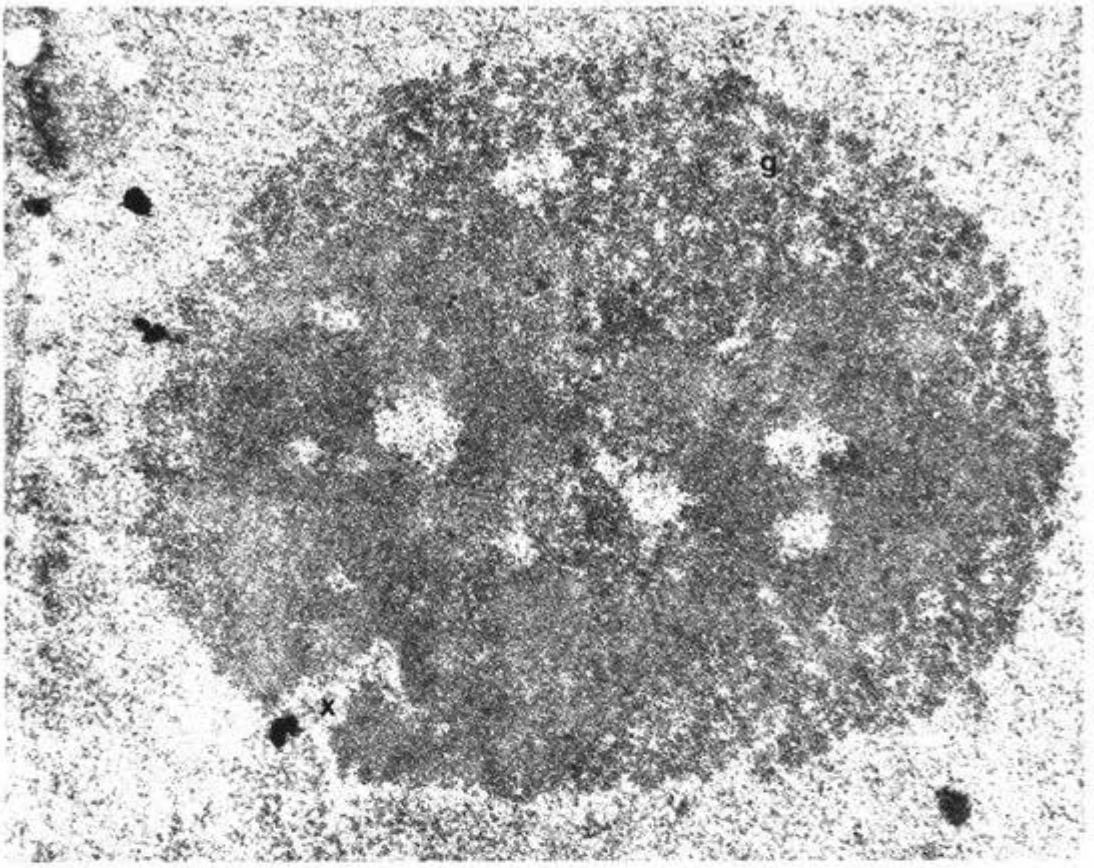
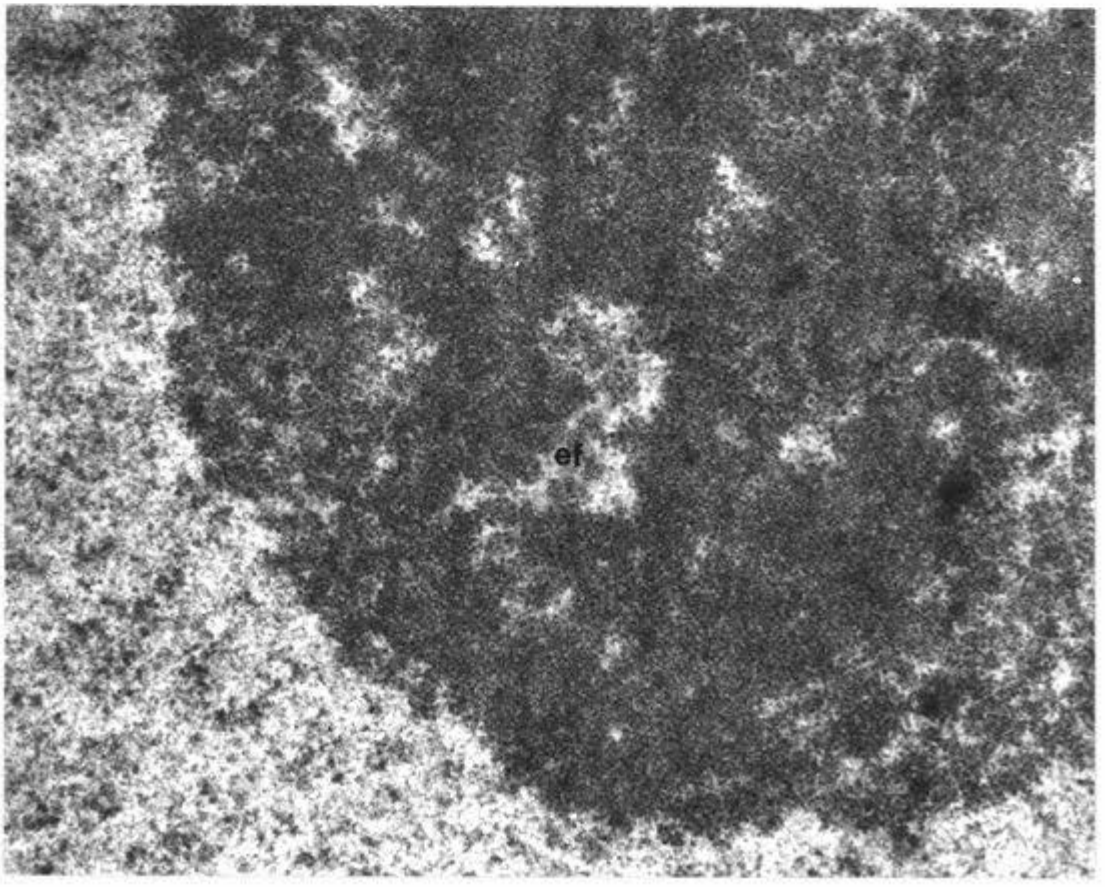

cy
n
p

Plate 4.6

A. The nucleolus of a cortical cell in the hypocotyl of a seedling after 16 hours of germination. The nucleolar diameter is 4.4 μm, one of the largest found during this study. The granular zone (g) is extensive and mainly peripheral. Nucleoli maintain a spherical shape with a defined outline. This would not be so if the granular material drifted randomly into the nucleoplasm. There is one nucleolar vacuole which is continuous with the nucleoplasm (x).
(X 22,500)

B. A part of the nucleolus shown in plate 4.5 A, showing expanded fibrillar material (ef) within lacunae.
(X 41,400)


g
x


ef

4.2 Ribosomes

Ribosomes are particles of ribonucleoprotein about 200 to 300 Å in diameter. The overall ribosome composition is 45 to 50% RNA and 50 to 55% protein. Immature ribosomal subunits derived from the nucleolus are transferred to the cytoplasm where they are further processed and assembled into polyribosomes. The details of this process are not fully known.

Although single ribosomes (monosomes) are found in cell cytoplasm, protein synthesis is carried out only by polysomes, as was first suggested by Warner et al. (1963).

Using the electron microscope, isolated polysomes are seen as extended arrays of ribosomes connected by an approximately 10 Å wide strand of presumed messenger RNA. The mRNA strand is rarely seen in sections of cells but the occurrence of discrete clusters of ribosomes in such preparations is considered to represent polysomes. Polysomes may be free in the cytoplasm or bound to endoplasmic reticuli (e.r.) in helical, spiral, or rosette configurations, forming rough e.r. The number of ribosomes per polysome varies considerably (Bonnett and Newcomb, 1965; Keyhani et al., 1971) and is presumed to reflect the length of mRNA and therefore the size of the polypeptide chain being synthesized.

The earliest time sampled in this electron microscope study of ribosomes in cortex and epidermal cells was 16 hours after the beginning of imbibition of water by the seeds. The 16 hour cytoplasm is densely packed with ribosomes as shown in plates 4.10 A and B. Some may be monosomes but most appear to be grouped either on e.r. or as free polysomes. Rough e.r., already fairly common by this stage, are bulgy or dilated in appearance and occur in short wavy lengths(plate 4.10 A). They are orientated randomly with respect to each other and to the wall.

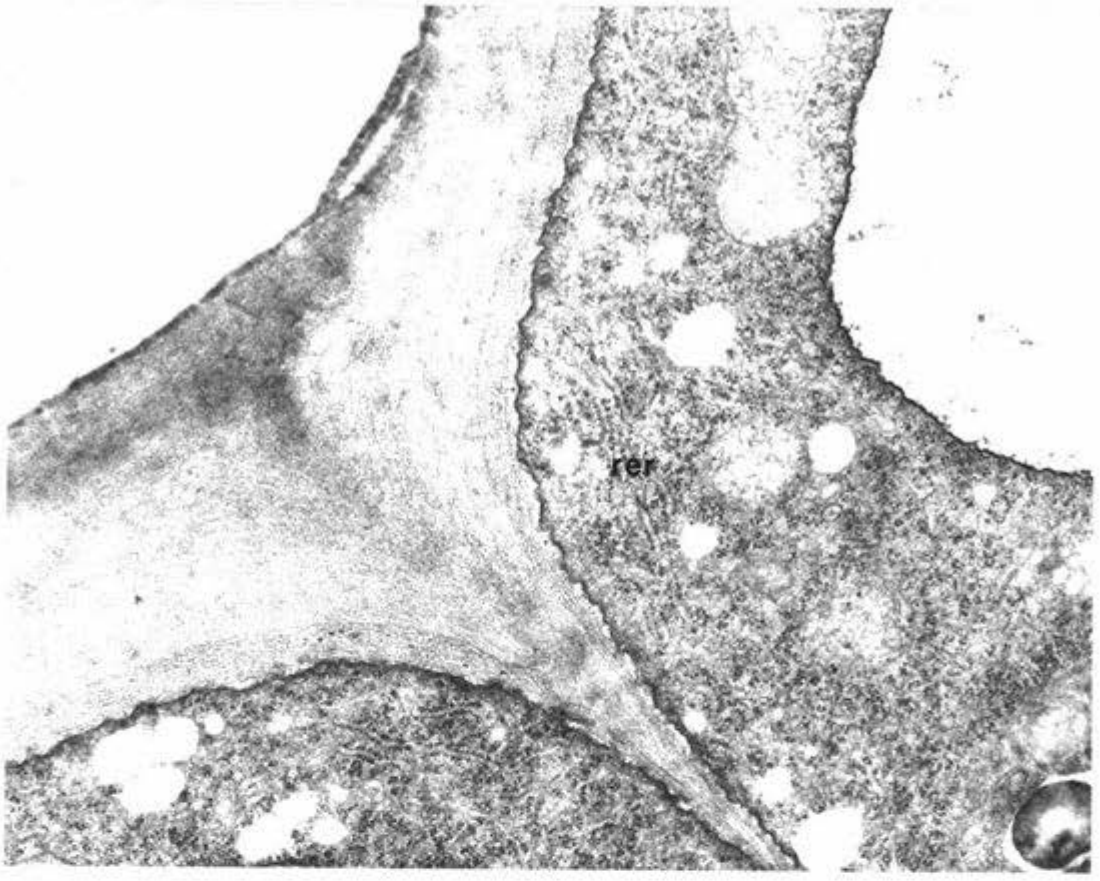
By one day, epidermal and cortical cells have started their first

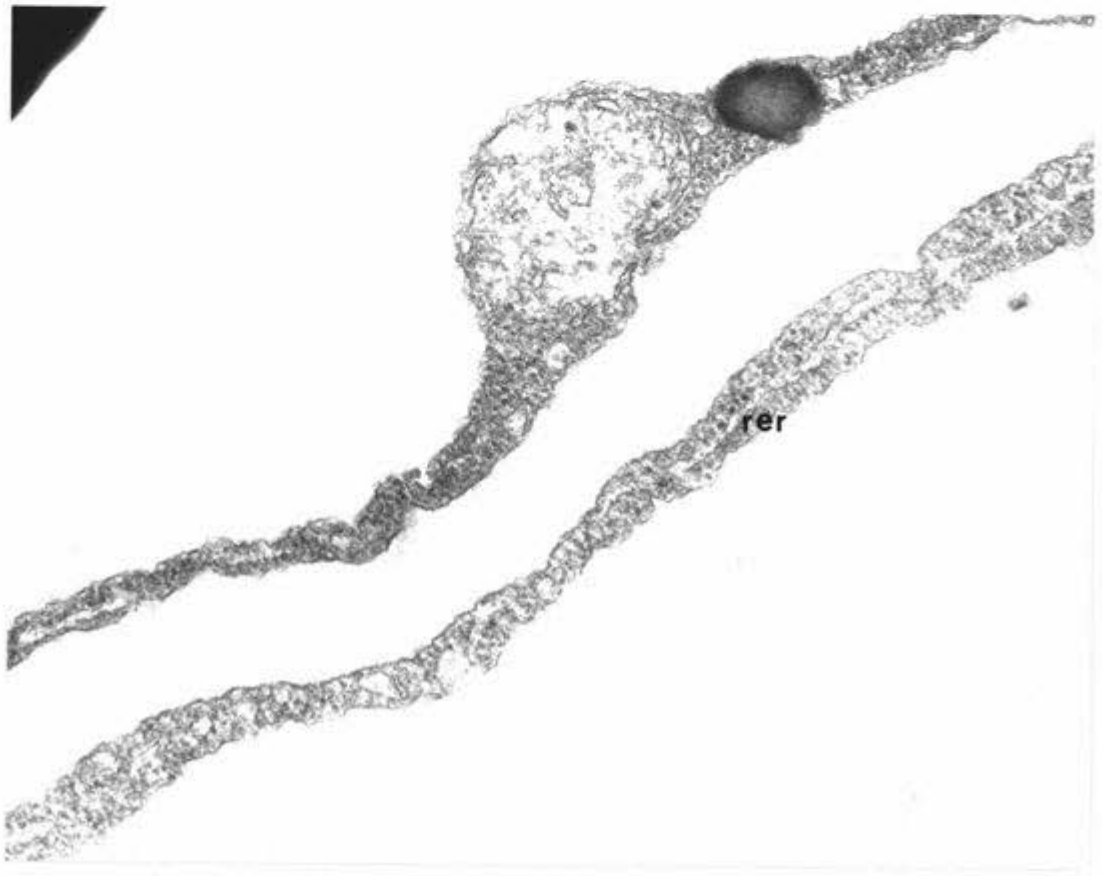
phase of rapid elongation; vacuolation is occurring and the layer of cytoplasm is becoming thinner, less than 2 μm in many cases. The cytoplasm is less densely packed with ribosomes and discrete polysomes are seen more clearly as in plate 4.12 A. Endoplasmic reticuli have a high number of ribosomes per length (plate 4.7 A) compared with the 16 hour tissue. There is a trend during this phase of early cell elongation for the cisternae of the e.r. to become straight, parallel sided, and to loose their dilated appearance. Longer continuous lengths of rough e.r. occur and they tend to be orientated parallel to the cell wall. Where rough e.r. are numerous they are found stacked in parallel layers. The decrease in the thickness of the cytoplasm probably forces the rough e.r. to assume the new orientation. Because of the quantity of rough e.r. relative to the cytoplasm thickness and wall area, the rough e.r. must lie in multiple layers. This pattern of ribosome arrangement continues in cortex and epidermis of region 3 up to 2 days, in region 2 up to 3 days and continues to at least 5 days in the top region (see plate 4.7 B). Over this time the trend is for the arrangement to change toward that found in the bottom region after 2 days and region 2 after 3 days. In cells of such tissue the layer of cytoplasm is very thin, often less than 0.15 μm, although it may be a little thicker in the cell corners. The ribosomes have less staining contrast with the rest of the cytoplasm because the cytoplasm contains a densely staining background material. This has been noted in maturing cells by other authors (Chaly and Setterfield 1975). At this late stage the predominant arrangement of ribosomes is in groups on the endoplasmic reticulum. Rough e.r. is seldom more than one layer thick in the cytoplasm, but this single layer occurs along a high proportion of the wall length seen in the thin sections (plate 4.7 B). A quantitative estimate of the amount of rough e.r. per cell compared with the earlier developmental

Plate 4.7

A. Epidermis cells of the top region of a 1 day old hypocotyl. Rough endoplasmic reticuli (rer) are numerous and they tend to be orientated parallel to the cell wall. As the cells expand and the layer of cytoplasm becomes thinner the rough e.r. assume this orientation. The outer wall of epidermal cells was always much thicker than the walls adjacent to other cells.
(X 22,500)

B. Cortex tissue from the basal region of a 4 day old hypocotyl. The layer of cytoplasm is very thin. Rough endoplasmic reticulum (rer) is still a notable feature of the cytoplasm of cells at this stage of development. As seen here, it often occurs as a single layer and in longer continuous lengths than in the cytoplasm of cells at an earlier stage. The mitochondrion in this micrograph appears to be connected to the cell wall. Other cell wall-associated structures are described in later plates.
(X 41,000)


rer


rer

stages has not been made so it is not known whether some rough e.r. actually break down or whether they spread more thinly over the expanding wall area of the cell. Non membrane-bound polysomes are still present but in very reduced density in the cytoplasm.

Distribution of ribosomes with time seems to be similar in both cortical and epidermal cells indicating that changes in ribosome distribution are not necessarily associated with cell division, as epidermal cells show the same pattern but do not divide, but is associated with other cellular synthetic processes being performed at this stage.

4.3 Golgi Apparatus

Golgi apparatus consists of stacked membranous cisternae from which membrane-bound vesicles arise. They are most commonly found in cells with a secretory function. Mollenhauer and Morré (1976) show that Golgi are frequent in primary root tip cells and root cap cells which are involved in secretion of root cap materials.

It was not a primary aim in this project to study Golgi and they were not seen often enough in electron microscope studies for a correlation to be made between their frequency and the stages of development of the cell in which they occurred.

Occasionally they occurred quite commonly. Plate 4.8 A shows a cortex cell from the upper region of a 2 day hypocotyl. Glogi were seen only rarely in tissue of the lower region after 2 days or any where below the top region after 3 days. Plate 4.8 B shows a Golgi body seen in a cortex cell in the middle region of a 5 day hypocotyl.

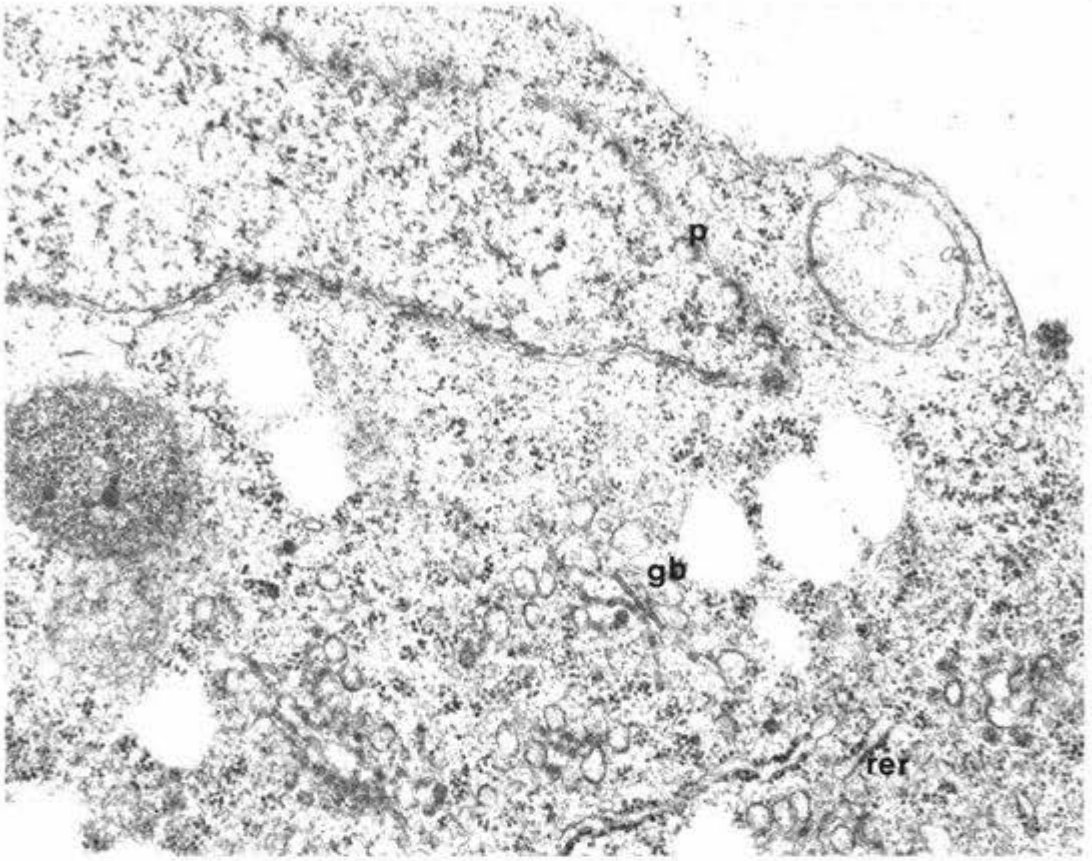
Vesicles similar to those from Golgi appeared to 'bud' off from rough endoplasmic reticulum in some cases (plate 4.8 A).

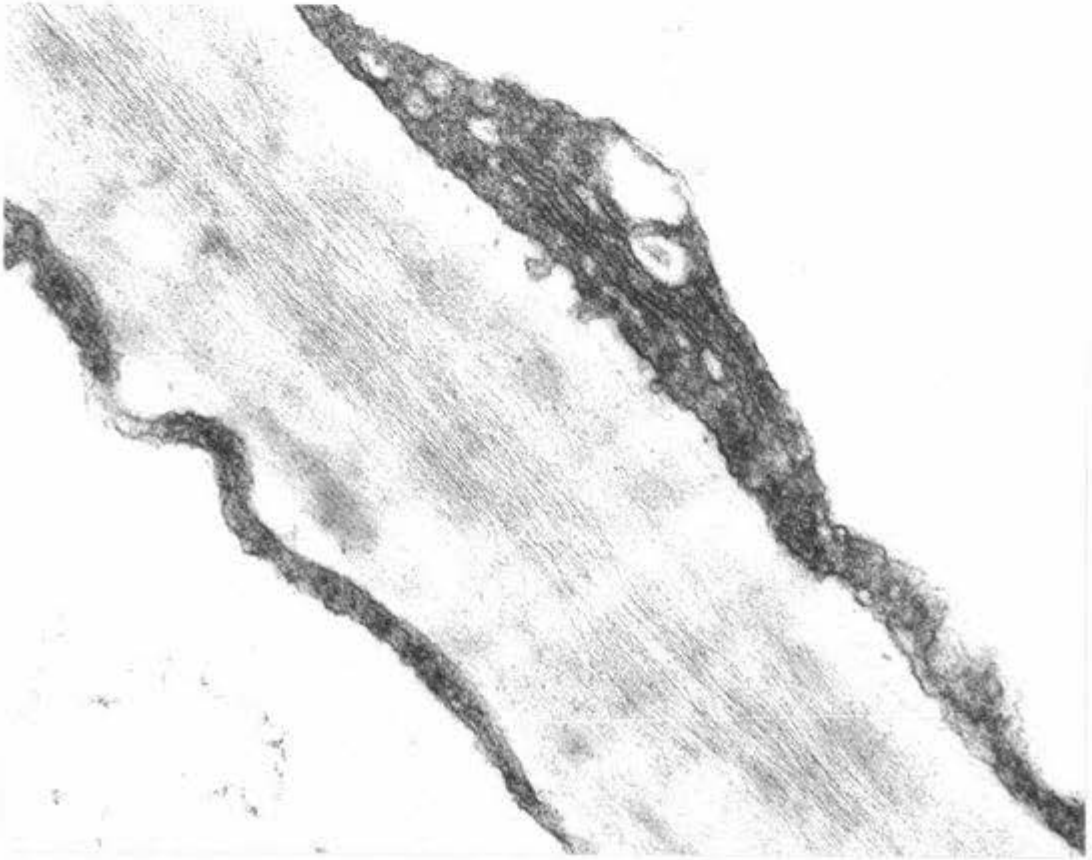
Gardiner and Chrispeels (1975) suggest an important role of the Golgi

Plate 4.8

A. A cortex cell from the upper region of a 2 day hypocotyl. This cell has numerous active Golgi bodies (gb). Rough e.r. (rer) have the wavy dilated appearance which was common in cells at an early stage of development. Pores (p) are frequent in the nuclear membrane.
(X 30,600)

B. Cortex cell in the middle region of a 5 day old hypocotyl. The cytoplasm is extremely thin. Golgi bodies, as shown in this plate, were very rarely seen in tissue at this age.
(X 55,000)


p
gb
rer



apparatus in the biosynthesis and the secretion of the cell wall glycoproteins of higher plants. This may be the function of the Golgi seen in the expanding lupin hypocotyl tissue.

4.4 Microtubules

Microtubules were observed only twice in this study; once in a cortex cell of 16 hour hypocotyl tissue (plate 4.10 A) and once in a developing hair cell (described in section 4.7) of a 2 day hypocotyl (plate 4.15 A). Both these cells were at a very early stage of development, before the cells had become vacuolated. The microtubules were mostly in bunches of 3 or 4 located in the cytoplasm close to the plasmalemma.

4.5 Plasmalemmasomes

Plates 4.9 to 4.11 show various membranous structures associated with the plasmalemma of cells at a range of stages of development. Although there are several rather different looking structures, they are grouped together because they are not often described and no definite function has been ascribed to any of them yet.

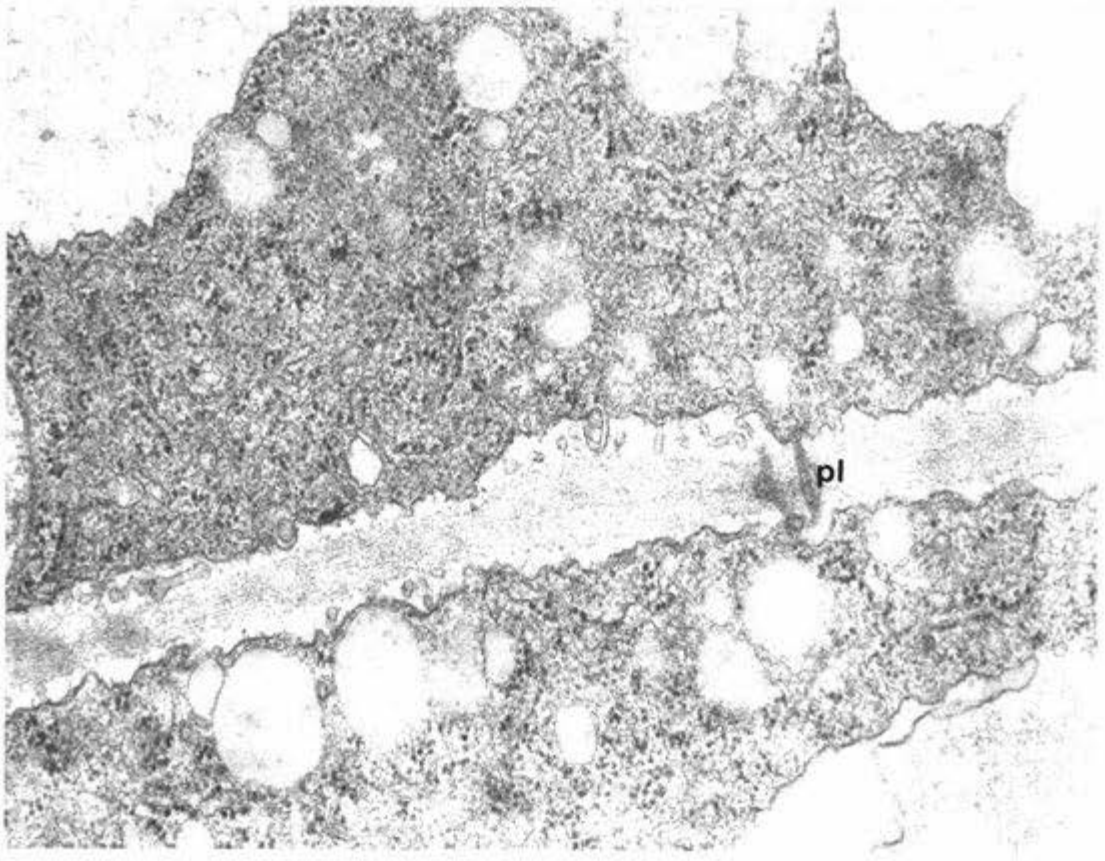
Plate 4.9 B shows a membranous structure within the cell wall, connected to the plasmalemma by plasmodesmata-like structures. Normal plasmodesmata are shown in plates 4.9 A and 4.10 A. Preston (1974) comments that,as cellulose synthetase enzymes are located in the plasmalemma, it may be significant that a number of workers have reported the presence in the cell walls of membrane systems clearly derived from the cytoplasm. A siting of synthetases on these membranes would harmonize with the reported synthesis of wall materials in the wall away from the plasmalemma.

In plate 4.9 A there are numerous tubular and vesicular membrane structures between the plasmalemma and cell wall. These are the type of

Plate 4.9

A. Longitudinal section through cortex tissue of a hypocotyl after 16 hours of germination. Plasmodesmata (pl) pass through the wall, connecting the two cells. There are numerous tubular and vesicular membrane structures between the plasmalemma and cell wall.
(X 41,400)

B. Cortex tissue in the top region of a 4 day old hypocotyl. A membranous structure in the cell wall is connected to the cytoplasm of adjacent cells via plasmodesmata-like structures. Rough e.r. (rer) are still present in the cytoplasm.
(X 55,000)


pl

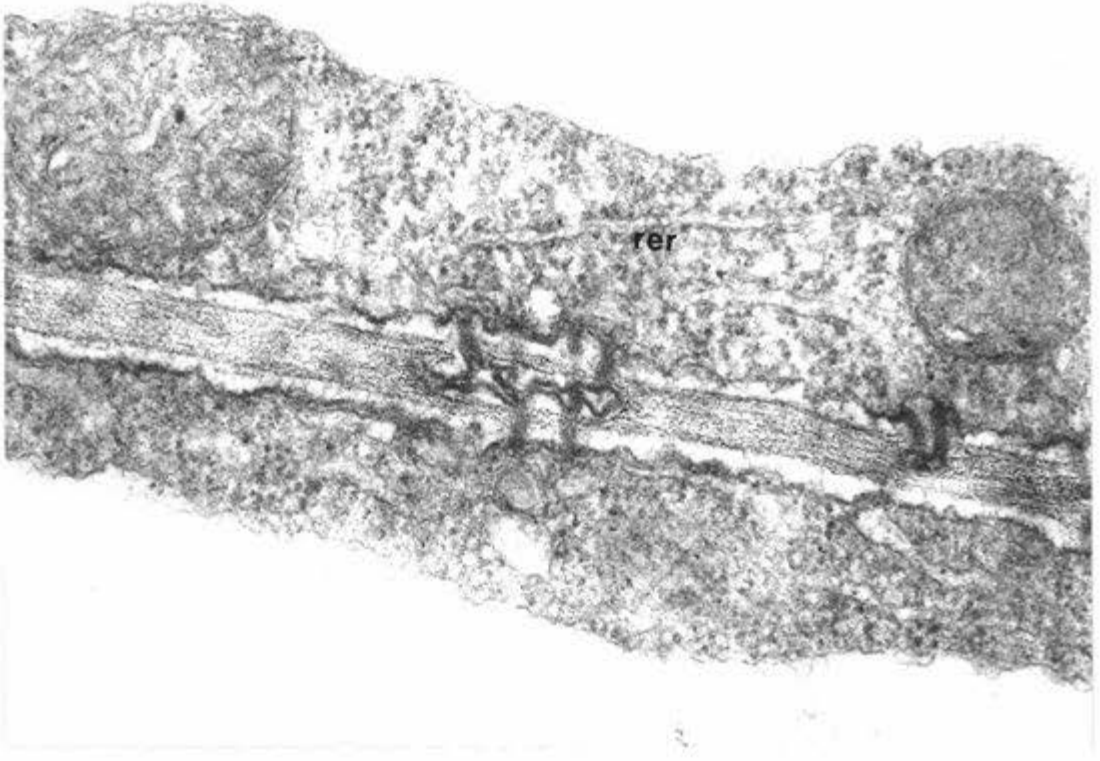
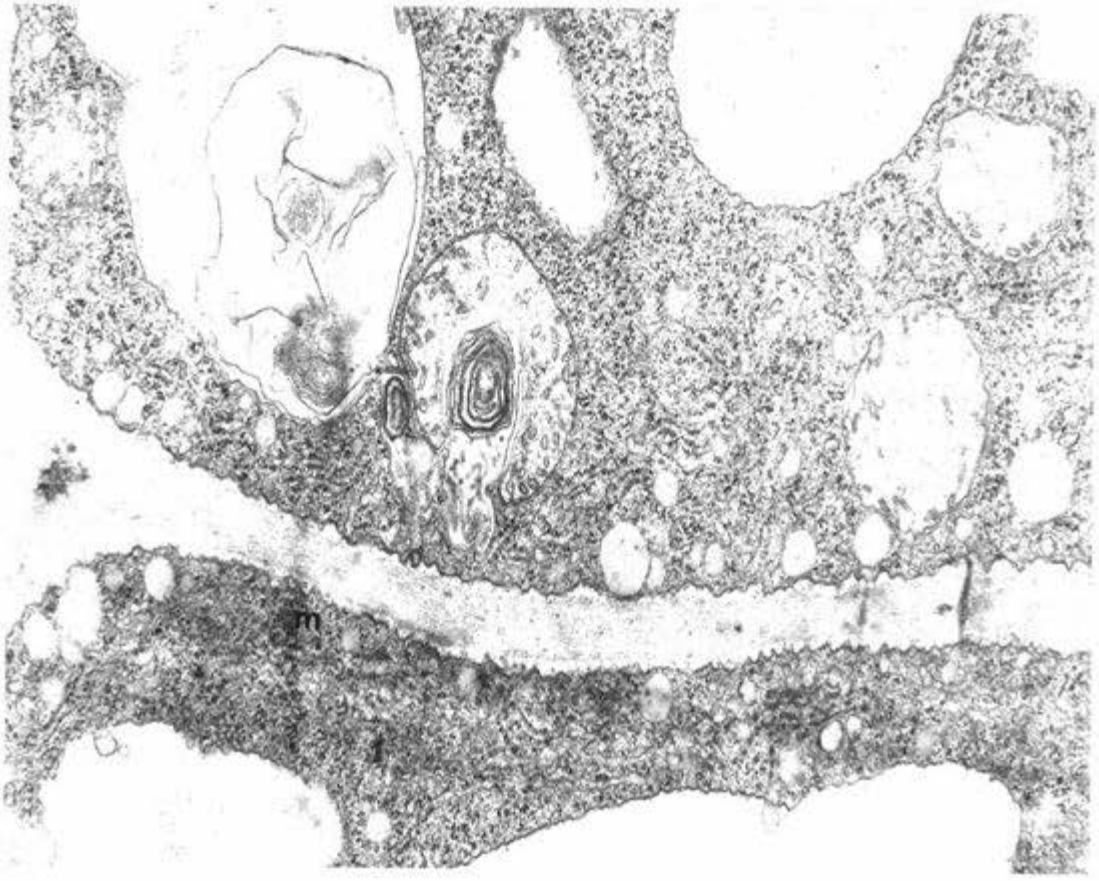

rer

Plate 4.10

A and B. Cortex cells in a hypocotyl 16 hours after the start of germination. The cytoplasm is packed with rough e.r. and polysomes. Rough e.r. have a dilated and wavy appearance at this stage. Microtubules (m) are present near the walls and plasmodesmata cross the wall in plate A. Plate B illustrates thick and thin regions of wall.

Both plates illustrate an unusual type of plasmalemmasome. It consists of a 'knot' of membrane surrounded by loose tubules or sheets of membrane. This structure appears to be in very close association with a mitochondrion.
(A X 22,500 B X 41,400)


m

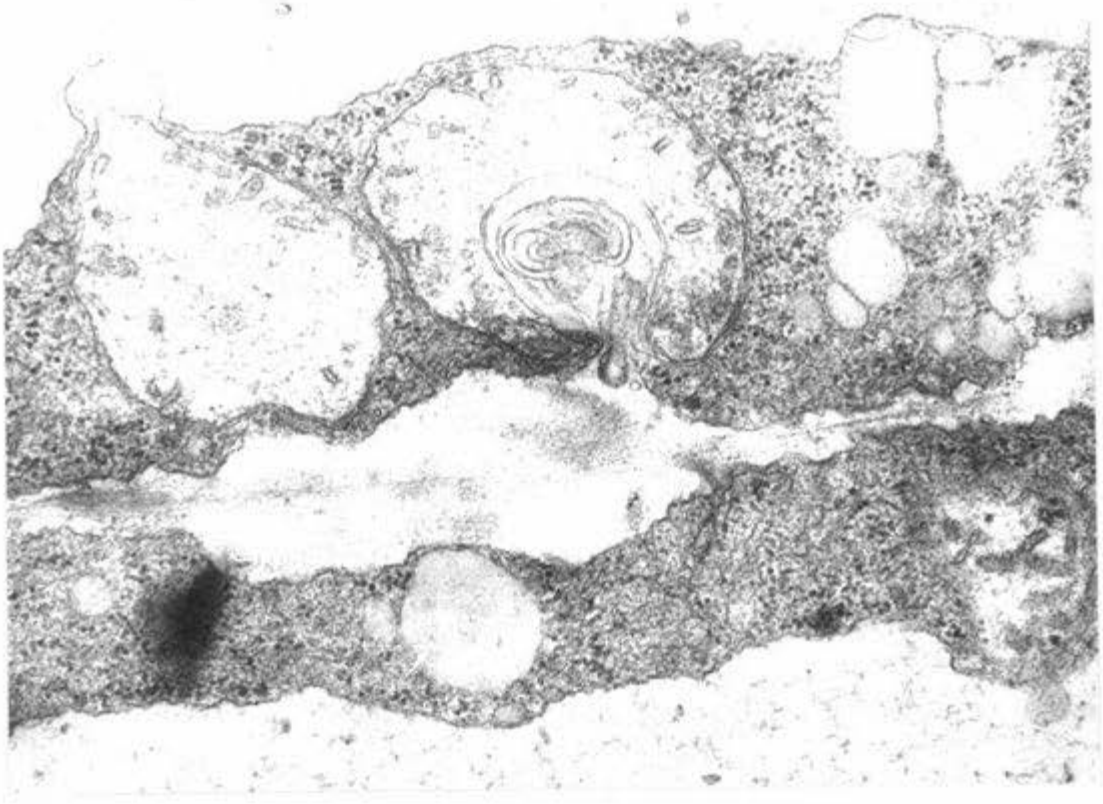
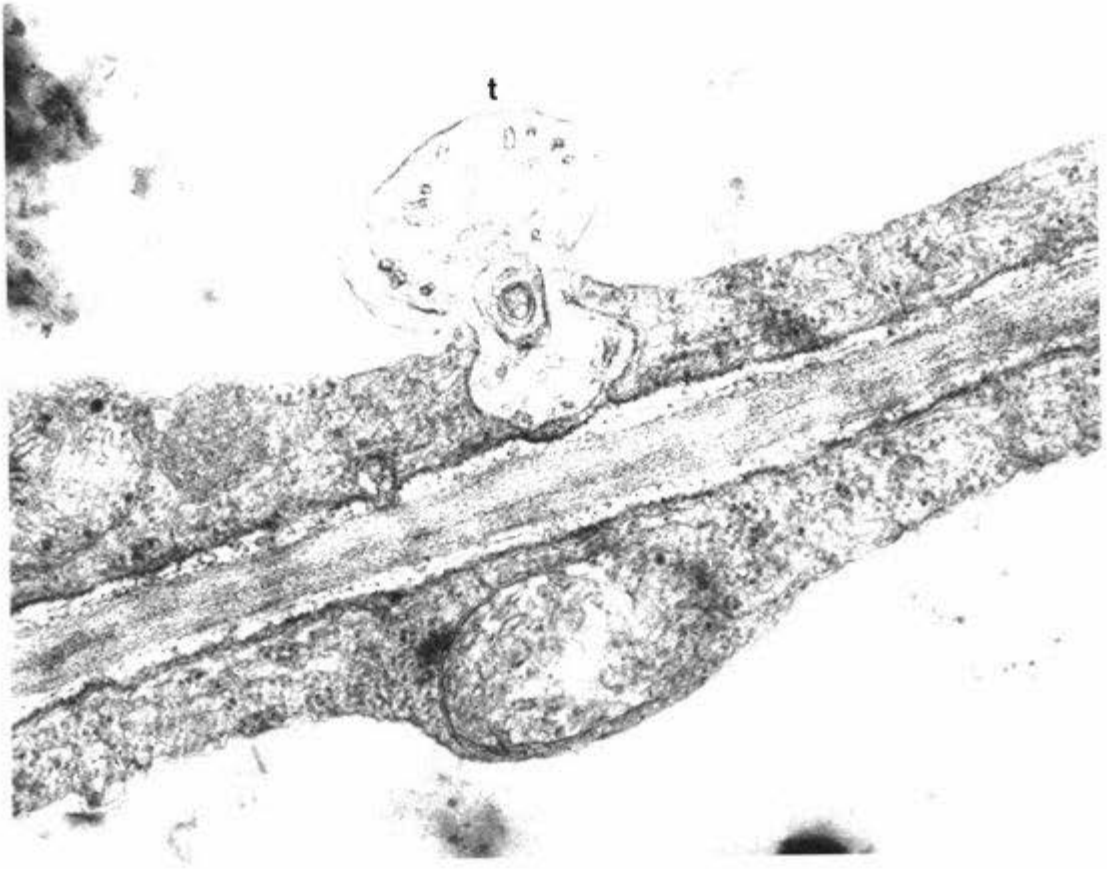
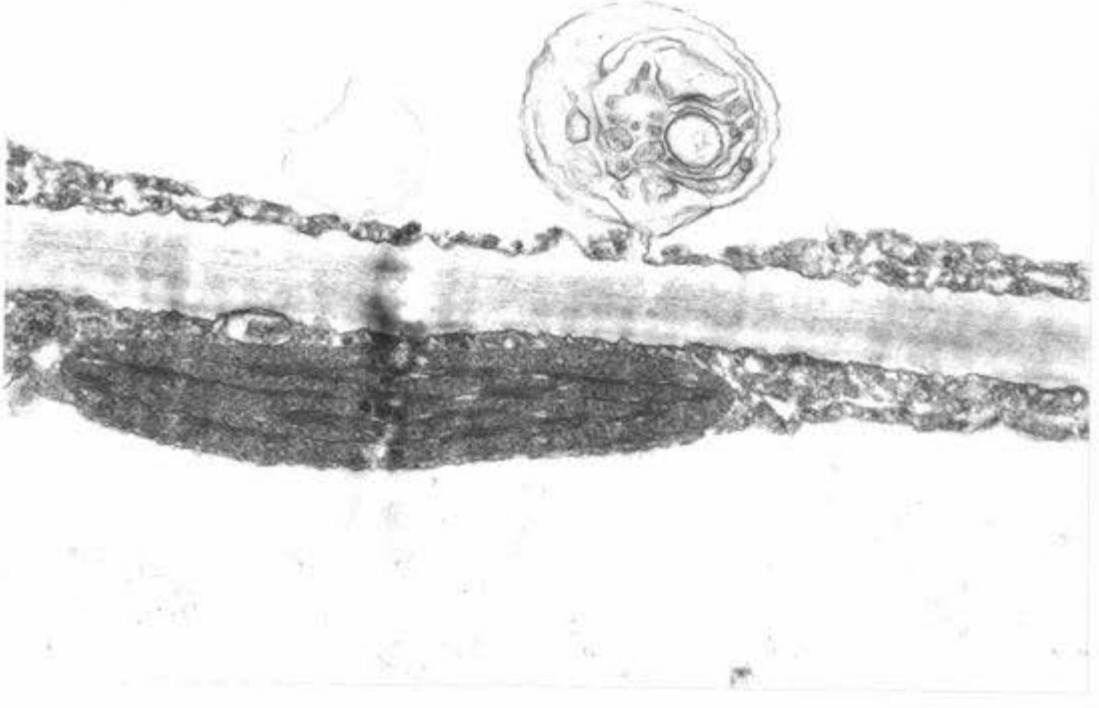


Plate 4.11

A. Epidermal cells in the basal region of a 3 day old hypocotyl. The plasmalemmasome shown is of a similar type to the ones in plate 4.10 but it is not associated with a mitochondrion. This plasmalemmasome protrudes into the cell vacuole and is surrounded by the tonoplast (t).
(X 41,400)

B. Longitudinal section through cortex cells in region 2 of a 5 day old hypocotyl. This shows a less structured type of plasmalemmasome. Possibly it is an artefact formed during fixation of the tissue. The layer of cytoplasm is extremely thin in a mature cortex cell like this and a structure like this could form as a result of disruption of tonoplast and plasmalemma.
(X 22,500)


t



plasmalemmasomes most commonly reported in plant cells and they have been reviewed by Marchant and Robards (1968). The structure shown in plate 4.11 A consists of a central 'knot' of membrane surrounded by loose tubules and sheets of membrane. A similar structure is shown in plates 4.10 A and B where it appears to be in very close association with a mitochondrion. This was found only in tissue sectioned 16 hours after germination. Further cytochemistry would be required to give positive proof that the organelle surrounding the membrane structure is in fact a mitochondrion. Possibly the synthesis of some cell wall components requires enzymes which are located within mitochondria. Further investigation of these mitochondrion-associated membrane structures could add to the theory of cell wall synthesis.

4.6 Cell Walls

Elongation of the lupin hypocotyl is a consequence of the longitudinal expansion of its component cells. In this study the thickness of walls of cortex and epidermal cells was measured.

The electron-microscopy study of the hypocotyl was not designed specifically for study of the cell wall, so the 120 measurements of wall thickness were made from micrographs taken primarily for describing other cytological features. However, although the sample numbers differ for each age, several conclusions can be drawn.

There was a range of thickness of cell walls, depending on the cell type and the position of the wall relative to other cells. The thickest walls were those bordering onto a space, such as the outer wall of epidermal cells and portions of cell wall surrounding intercellular spaces in the tissue. These walls also showed the greatest variation in thickness. The common wall between adjacent cortex cells or between cortex and epidermal

cells was narrower and more constant in width than the thicker space-bordering portions of wall.

Figure 4.1 shows the width of the thinner common walls between either adjacent cortex cells or cortex and epidermal cells. The mean and standard error of the mean for each age are shown. The wall width increases slightly from 0.23 μm at 16 hours to 0.28 μm by 2 days. Between 2 and 3 days the wall width decreases to 0.18 μm. Two to 3 days is also the time of the peak relative rate of increase in hypocotyl length. Perhaps at this stage the rate of wall expansion exceeds the rate of synthesis of wall material. By 4 and 5 days the wall width has increased again to 0.4 to 0.5 μm and the relative rate of expansion is lower at this time. The average wall width approximately doubles between 16 hours and five days. The wall thickness in a particular cell probably depends on the relative rates of cell wall expansion and synthesis of wall material. (Synthesis of wall materials may be occurring at a maximum rate from the start of germination up to 5 or 6 days.)

The cell walls bordering intercellular spaces were wider than common walls between adjacent cells (plate 4.12A). This thickening of walls in cell corners may be the formation of collenchyma cells. Although there was high variation within an age group, the trend was for these walls to increase in thickness with age. The range of wall widths found at each age is given in table 4.2.

Table 4.2 Thickness of Walls Adjacent to Intercellular Spaces

Age	Mean Width - μm	Range of Widths
16 hours		0.51 (one value)
1 day	0.95	0.6 - 1.1
2 days	1.10	0.8 - 1.3
3 days	1.10	0.7 - 1.5
4 days		no values
5 days	0.86	0.7 - 1.4

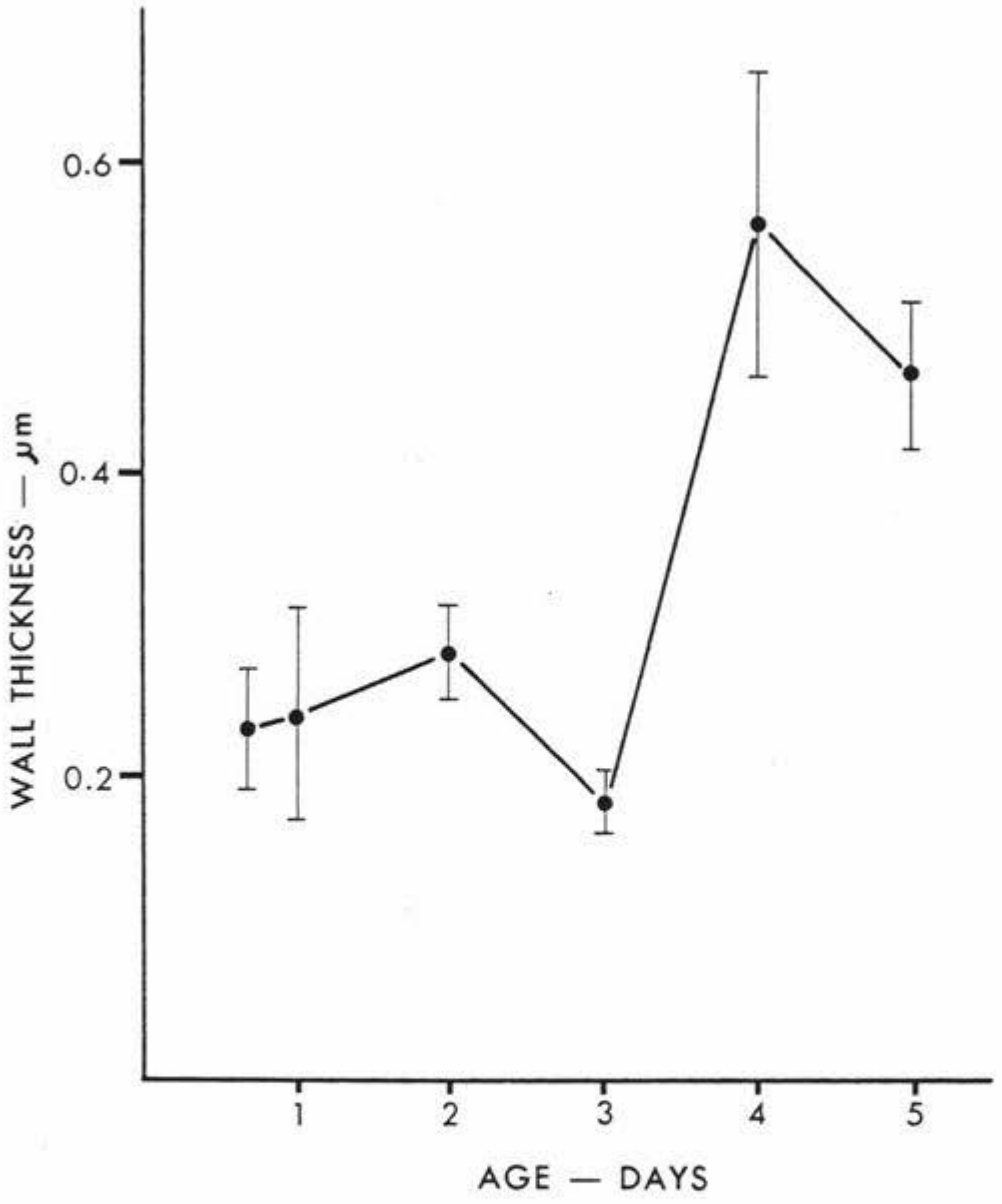


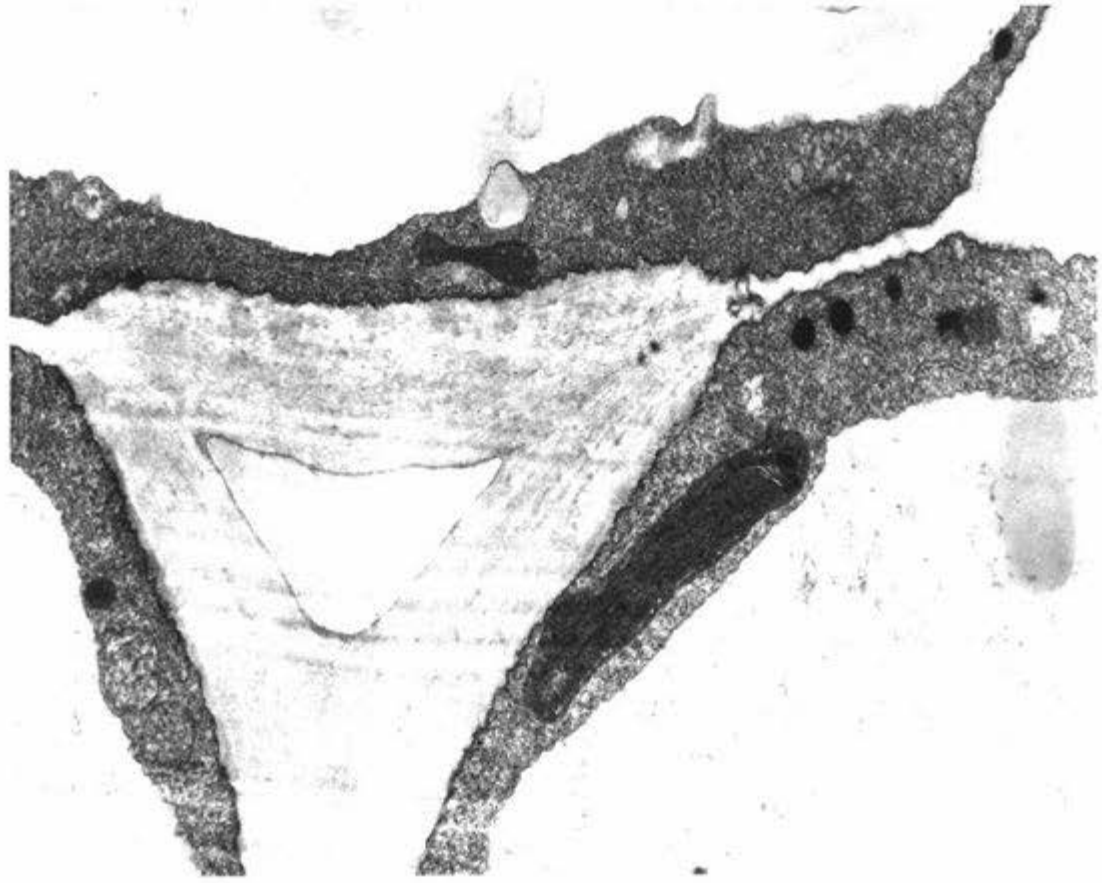
Figure 4.1

The mean width of the common walls between either adjacent cortex cells or cortex and epidermal cells. The standard error of each mean is indicated.

Plate 4.12

A. Cortex cells in region 2 of a 3 day old hypocotyl showing the contrast between the thick wall surrounding an intercellular space and the thin common wall between two cells.
(X 13,500)

B. Cortex cells in the basal region of a 4 day old hypocotyl showing the protrusions of the wall into the cytoplasm which were common in mature cortex cells.
(X 30,600)



The outer walls of epidermal cells are also very thick even from an early age. The measurements are given in table 4.3.

Table 4.3 Thickness of Outer Walls of Epidermal Cells

Age	Mean Width - μm	Range of Widths
1 day	0.83	0.5 - 1.0
2 days	1.10	0.8 - 1.8
3 days		1.65 (one value)
4 days		2.46 (one value)
5 days		no values

There must be a high rate of synthesis of wall material in the epidermal cells in order for them to maintain this thick outer wall during the phase of rapid expansion. The other walls tend to be quite thin, so wall material is probably laid down preferentially on the outer wall. Possibly microtubules have a role in directing material to the outer wall.

Some unusual patterns of wall thickening were noted. Plate 4.10 B shows part of a common wall between cortex cells in one day tissue, which have thick and thin regions of wall. In older tissue, the wall quite often had many small protrusions into the cytoplasm (plate 4.12B).

4.7 Hair Cells

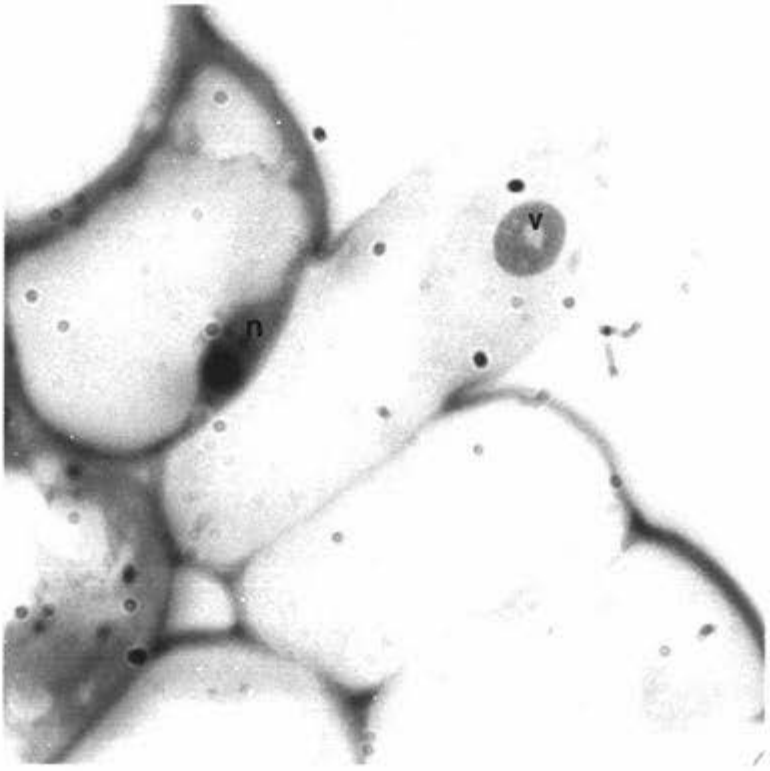
The hypocotyl of the lupin seedling is covered with stiff hairs about 0.5 mm long. Hair cells are first distinguishable from other cells of the epidermis at about 1 day after the start of germination. Most epidermal cells are well vacuolated at this age and have a thick outer cell wall, but hair cells are packed with dense cytoplasm and the outer cell wall is relatively thin. The hair cell starts to protrude from the surface of the epidermis and may be quite long by three days. Plate 4.10 shows hair cells at different stages of development, but both on the surface of a 3 day old hypocotyl.

It is interesting that a single hair cell may be at a very different

Plate 4.13

A and B. Light micrographs of hair cells on the surface of a 3 day old hypocotyl. As the hair cell grows, the nucleolus moves out into the hair. The hair cells are at a very different stage of development from their adjacent epidermal and cortex cells. The adjacent cells are highly vacuolated and the nucleus (n) in plate A is small with a densely staining compact nucleolus. The nucleoli in the hair cells both have a large central vacuole (v) as well as several smaller ones.

(A and B X 1300)


v
n

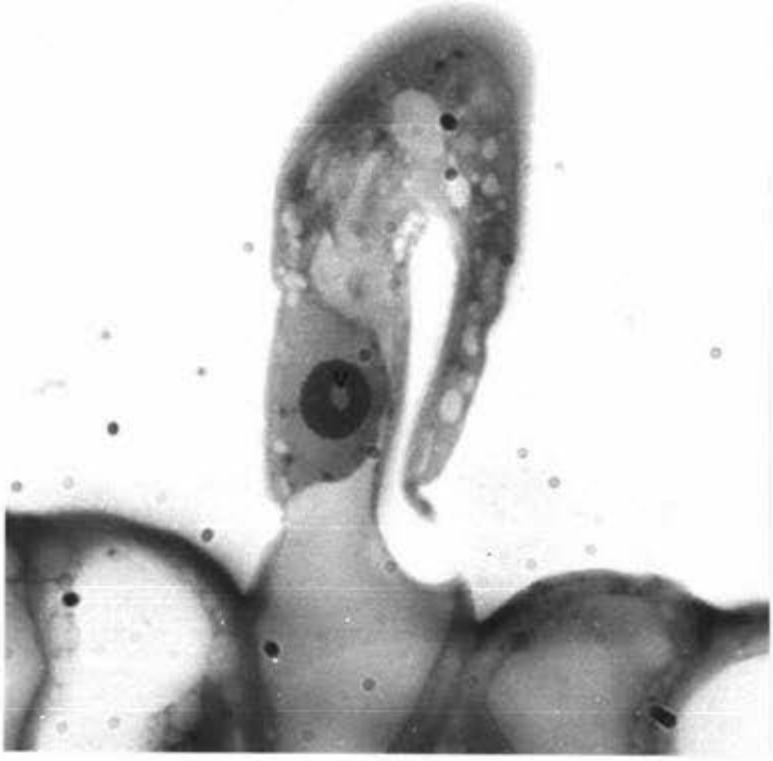

v

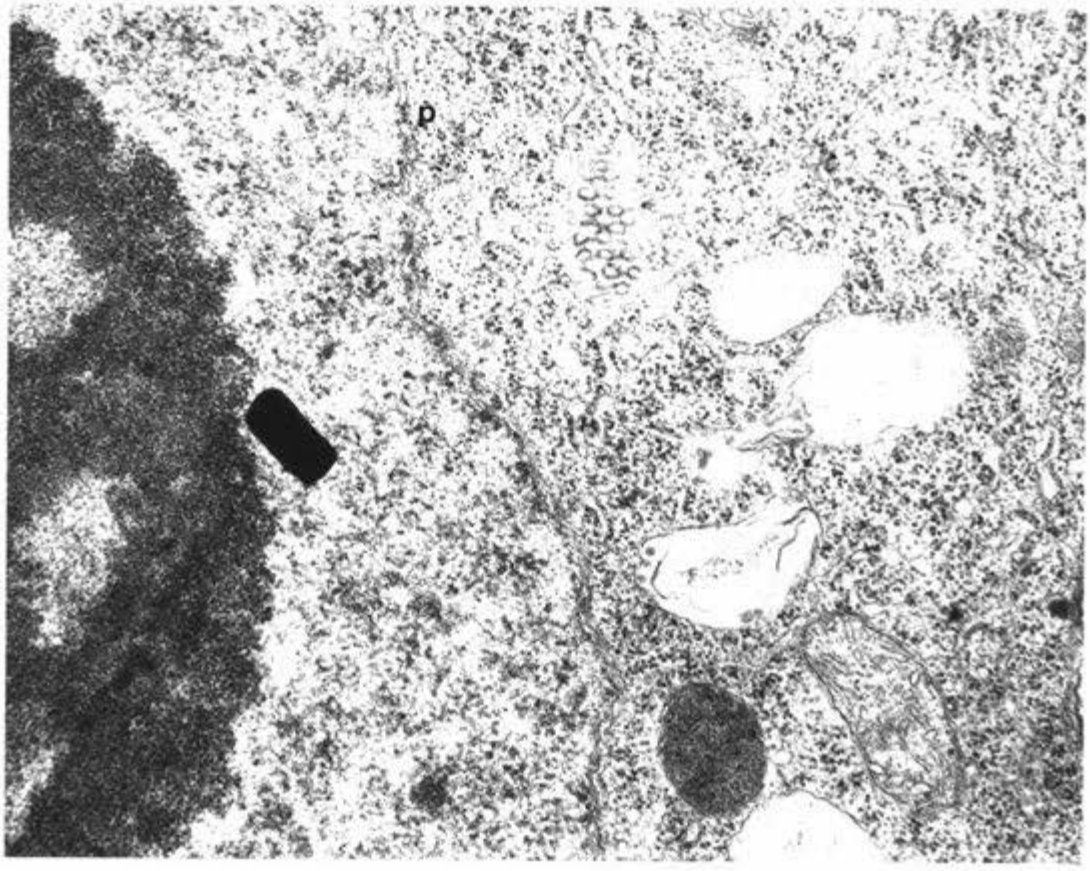
Plate 4.14

A. A longitudinal section through a hair cell from region 2 of a 2 day old hypocotyl. Pores (p) are frequent in the nuclear membrane. The cytoplasm is packed with wavy, dilated rough e.r. and polysomes.

(X 30,600)

B. An unusual microtubular structure is seen in the above plate and it is shown here viewed at a higher magnification. The actual tubules measure about 540 Å in diameter. A possible function for this structure may be to push the nucleus out into the hair as the hair cell expands.

(X 128,000)


p

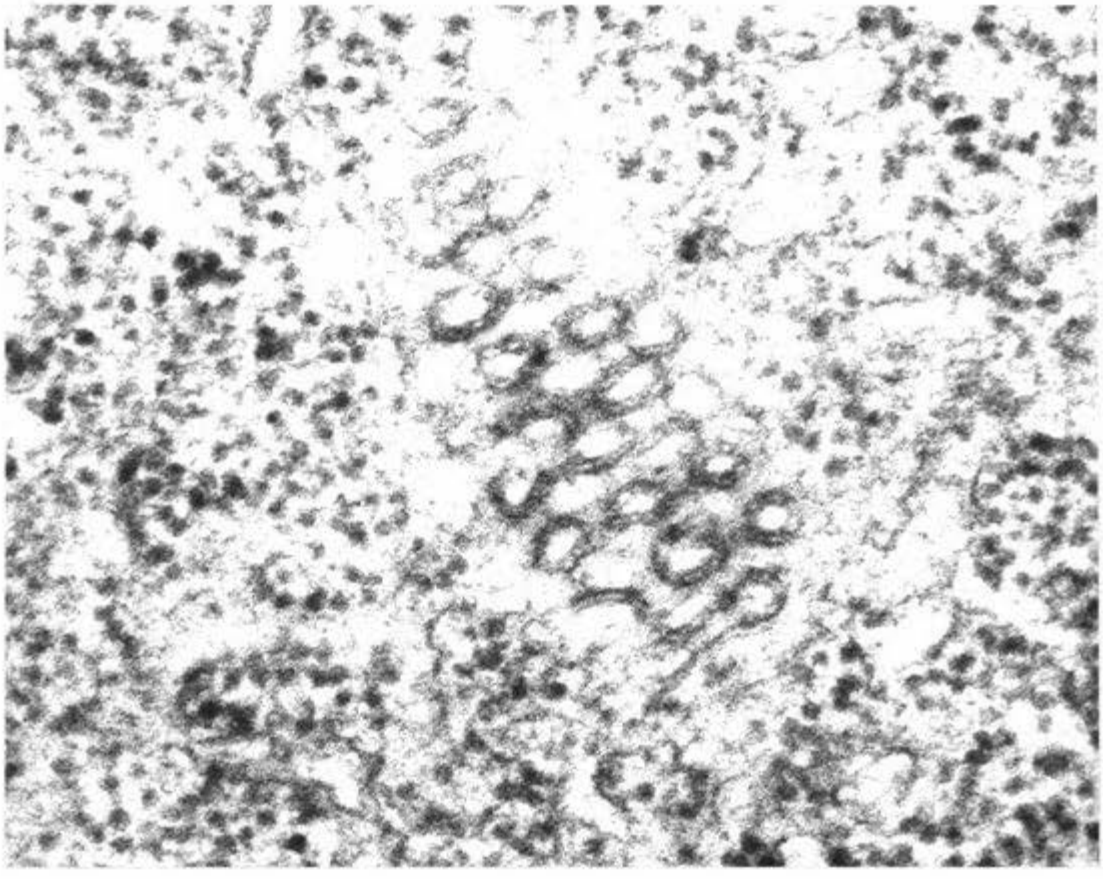
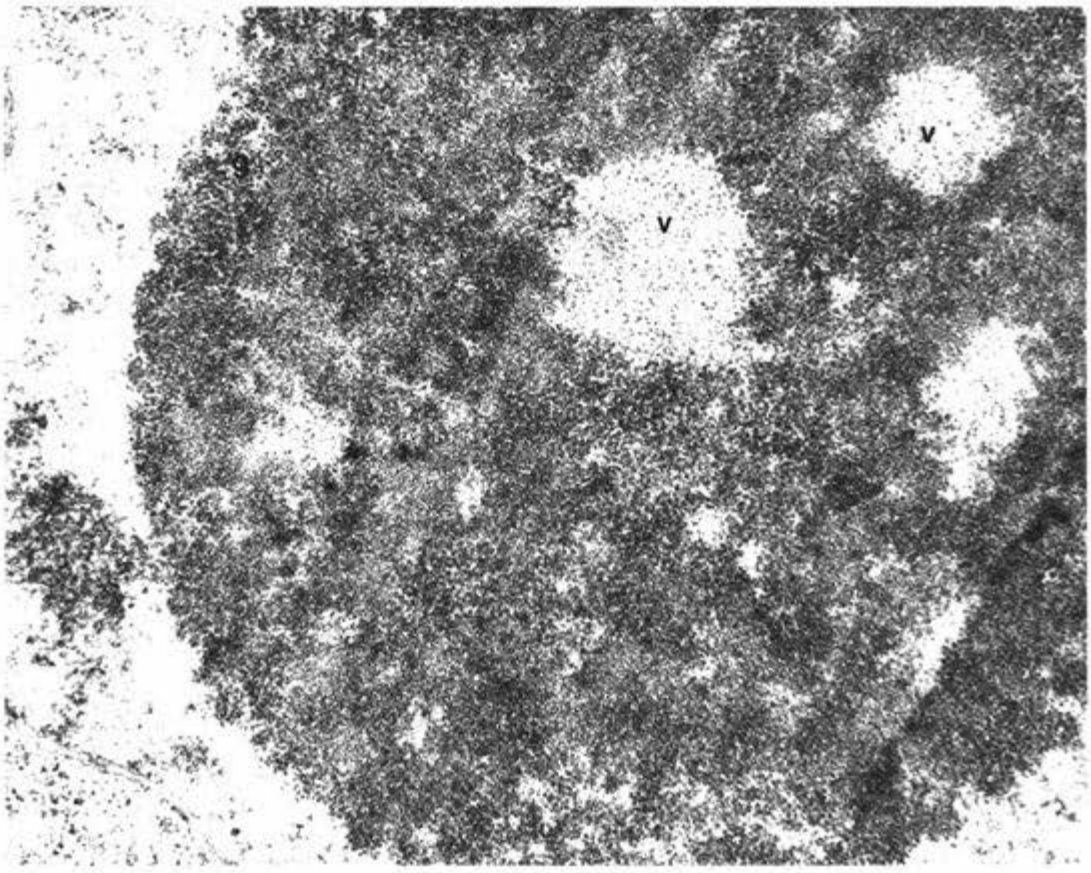
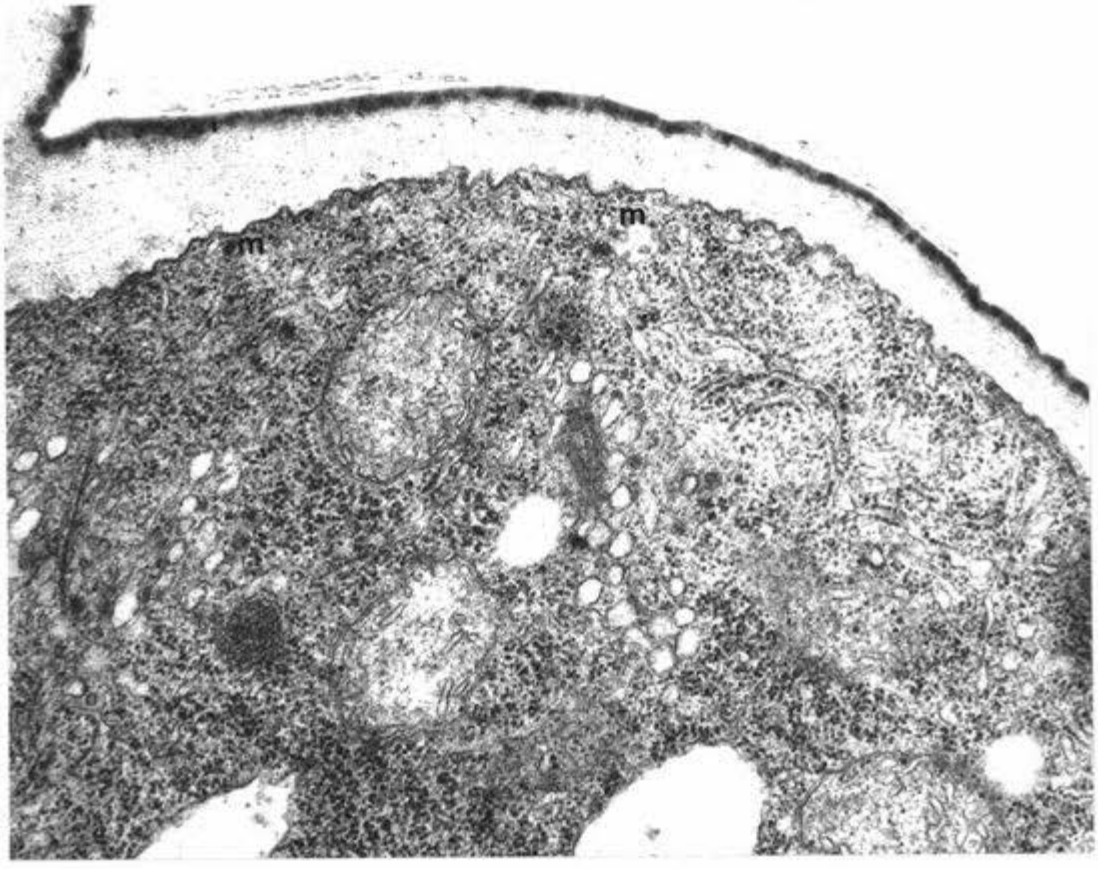


Plate 4.15

A. This is the nucleolus of the hair cell illustrated in plate 4.14. The nucleolus is in an expanded state with many vacuoles (v) and large granular zones (g). There is a connection between the nucleolus and some electron dense material in the nucleoplasm; possibly material expelled from a nucleolar vacuole, or condensed chromatin connected to the fibrillar portion of the nucleolus. This nucleolus has a diameter of about 4.1 µm.
(X 30,600)

B. The outer portion of this same hair cell. The hair cell is just starting to protrude from the surface of the epidermis. The outer cell wall is thin compared with the outer walls of other epidermal cells. Microtubules (m) occur in groups along this outer wall. Golgi bodies are active in this cell.
(X 30,600)


v
v


m
m

stage of development from all its adjacent cells. The hair cell illustrated in plates 4.14 and 4.15 has many of the features typical of an active meristematic cell. The nucleolus is in an expanded state with many vacuoles and large granular zones. Pores are frequent in the nuclear membrane and the cytoplasm is densely packed with ribosomes and rough e.r. Golgi bodies were found more frequently in hair cells than in other epidermal or cortex cells at a similar stage of development. Microtubules were numerous along the outer wall of young hair cells.

As the hair cell grows, the nucleus moves out and stays close to the growing region of the cell (plate 4.13). An unusual microtubular structure is shown in plate 4.14 and it is possible that its function is to move the nucleus into the hair as the hair cell expands.

Once the hair is formed, the cell becomes highly vacuolated like the adjacent epidermal and cortex cells.

4.8 Conclusions

Cytological changes were observed in cells of epidermal and cortex tissue during germination of the lupin seedling.

The nucleus has the important role of coordinating and directing activities in the cell. In this study a good correlation was found between cell size and certain features of the nucleus.

The nucleus has a central location in cells of the early germinating seedling but usually assumes a peripheral position as the central vacuole forms in the cell. The diameter for nuclei of one day cortical tissue was very close to the value found by Chaly and Setterfield (1975) for nuclei of cells within 1 mm of the root apex of Pisum sativum. The actual volume of the nucleus may not be as important as the surface area available for interaction between the nucleus and cytoplasm. The nuclear membrane was

found to show varying degrees of convolution in cortex tissue of seedlings up to three days old. A high frequency of pores in the nuclear membrane was found in tissue up to 2 days old. These two factors suggest that during the first two days of seedling germination there is extensive interaction between the nucleus and cytoplasm in cells of the epidermis and cortex.

Nucleoli were found to have their maximum diameter within the first day of germination. The decrease in size after one day was due to condensation of vacuolar regions and loss of granular regions. The nucleolus is at its maximum size at the same time as the nuclear membrane is convoluted and has a high frequency of pores.

In the introduction the findings of several electron microscope studies of ribosomes were described and the conclusion was drawn that the number of ribosomes formed in meristematic and very early elongating cells is sufficient to sustain subsequent expansion growth. The results of this study support the generalization.

The cell wall width study indicated that, at the peak of cell expansion, the average wall width actually decreased, suggesting that the rate of cell expansion is relatively faster than the rate of synthesis of wall materials during this time.

CHAPTER FIVE RNA IN THE LUPIN HYPOCOTYL

Ribosomes in the cytoplasm are necessary for protein synthesis to occur. The frequency of ribosomes relates to the level of synthetic activity in the cell at that time. In chapter 4 the abundance of ribosomes in the cytoplasm of the different cell types at a range of ages was compared. The ultrastructure of nucleoli was also studied and this gave an indication of the RNA synthetic activity of the cells in which they were contained. Changes in the total amount of RNA in whole hypocotyls of different ages is investigated. This gives the RNA content averaged for all cell types at all stages of development present in hypocotyls of that age.

The actual RNA content at any time depends on the relative rates of synthesis and breakdown of ribosomes, so rates of RNA synthesis are studied in conjunction with the actual RNA content of hypocotyls.

Determination of RNA content of the embryo hypocotyl in the seed allows the proportion of ribosomes formed during seed formation and following germination to be deduced.

5.1 RNA Content

The RNA content per hypocotyl was determined by averaging the results from the 3 replicates at each age. These results are shown in figure 5.1 A.

The embryo hypocotyl in the seed contains more than half the RNA that is present at any stage subsequent to germination. From 0.5 to 1.5 days, the RNA content increases rapidly from 0.8 to 1.3 mg per hypocotyl. From then the RNA content slowly declines until by 5 days it has again reached the level found in the seed.

Possibly the initial rapid increase in RNA content occurs so that all new cells formed during the peak of cell division in the cortex and

vascular tissue at 1.5 days, contain an adequate complement of ribosomes for the period of high synthetic activity occurring at this time. After 2 days, the cells of the vascular tissue have the greatest volume of cytoplasm and a high level of cellular processes occurring. It is likely that the decline in total RNA per hypocotyl from 2 to 5 days is the result of breakdown of ribosomes in the cortex and epidermis without equal synthesis of new ribosomes.

5.2 RNA Synthesis

In this study, RNA synthesis is determined by comparing incorporation of ^{32}P into RNA, during a 1.5 hour incubation period, with the total amount of ^{32}P absorbed by the tissue. This is expressed as a ratio of counts in RNA to counts in tissue, and is shown in figure 5.2. This analysis does not give quantitative rate of RNA synthesis but shows how the average rate of RNA synthesis changes with age of the hypocotyl.

The apparently high rate of RNA synthesis at 0.5 days may result, in part, from the fact that the germinating seed is still rapidly imbibing water at this stage (the hypocotyl is about 25 % dry matter by weight) and, in effect, the specific activity of label in the tissue is greater than at the later times when tissue is less than 10 % dry matter by weight (figure 3.4).

The rate of synthesis declines gradually from 1.5 to 5 days. Probably RNA synthesis continues in the vascular tissue so that the ribosome content of vascular cells is maintained. If the RNA content of vascular tissue stays at a constant level, then , in figure 5.1, the rise in RNA content from 0.5 to 1.5 days, followed by the gradual decrease till 5 days, results from rapid RNA synthesis in all cell types up to 1.5 days, followed by cessation of synthesis in the cortex, pith and epidermis (excluding

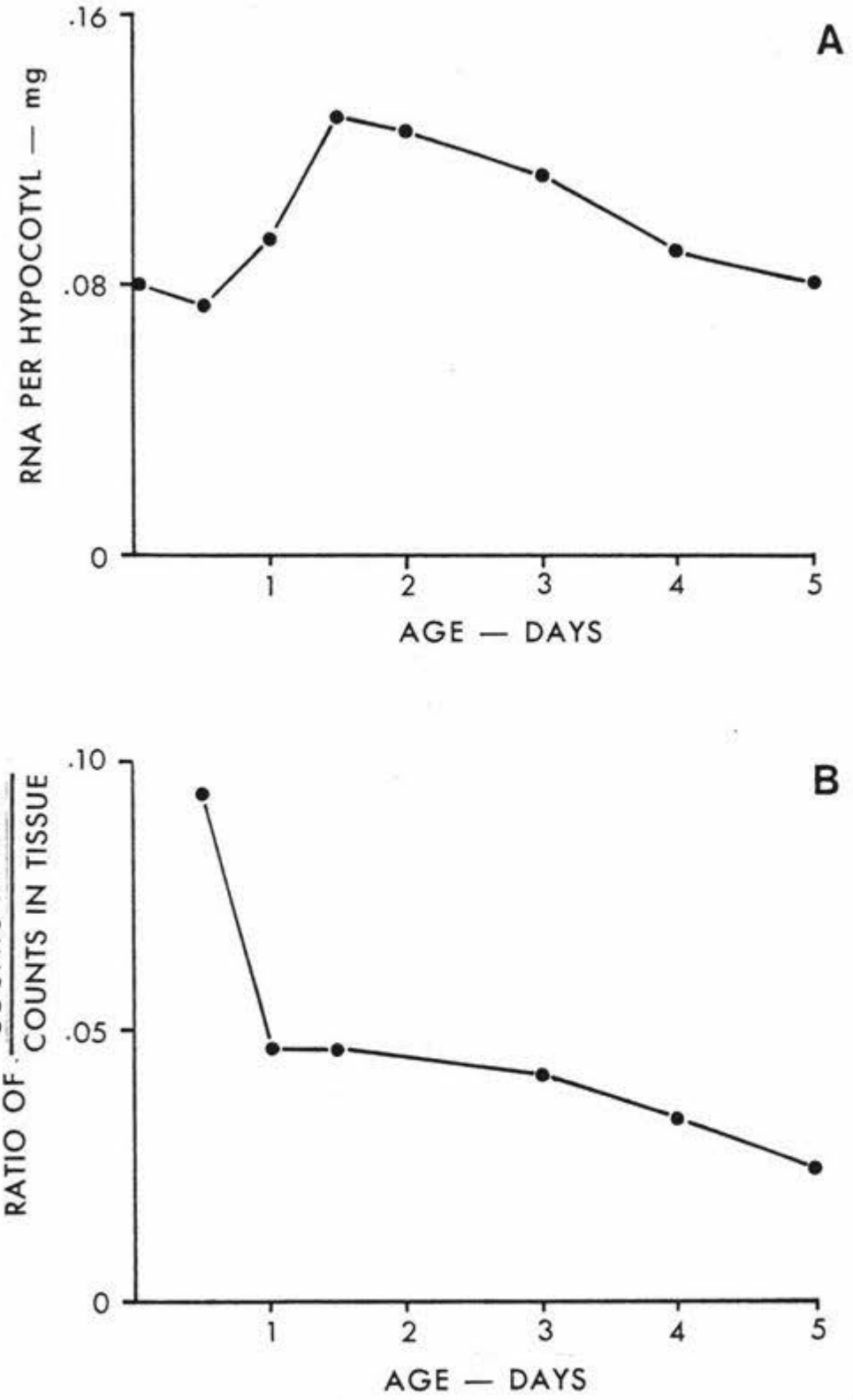


Figure 5.1

A. The mean RNA content per hypocotyl. Values for the embryo hypocotyl in the dry seed, up to the hypocotyl of the 5 day old seedling are given. B. Change in rate of RNA synthesis during seedling growth. (See section 5.2 for further explanation.)

hair cells) and only breakdown of ribosomes occurs in these tissues.

5.3 Discussion

The results, that RNA synthesis is greatest at 12 hours after the initiation of seed germination and slows down rapidly after 2 days, and that RNA content of a hypocotyl increases rapidly from 0.5 to 1.5 days, fit into the pattern of events indicated by other workers using the same approach, and also with results on nucleolar size and ribosome density as indicated in sections 4.1.5 and 4.2.

In the introduction, results of several studies on changes in RNA content and rates of RNA synthesis are described, including Wright's (1961) result that RNA content increases 275 % from 48 to 72 hours and only 31 % from 48 to 72 hours in the coleptile of germinating wheat embryo. Spiegel et al. (1975) studied transcription of ribosomal and messenger RNA's in early wheat embryo germination and found that rRNA synthesis increases steadily from 6 hours, reaching a peak at 16 to 18 hours in embryos germinating at 26°C.

Results presented here on RNA synthesis show that in this study, as in others described in the introduction, the time of peak RNA synthesis coincides with the time when nucleolar diameter is largest. It is shown in section 4.1.5 that nucleolar diameter, at least in cortex and epidermal tissue of the hypocotyl, reaches its peak during the first 24 hours of germination and declines after this time.

In the electron microscope study of cortical and epidermal tissue of the hypocotyl after 16 hours of germination, it was noted that the cytoplasm was densely packed with ribosomes (section 4.2). This indicates the presence of many ribosomes but not the time of their synthesis. Results on RNA content in figure 5.1 show quite a high RNA content in

the hypocotyl of the ungerminated seed so it is likely that a reasonable proportion of the ribosomes seen in 16 hour tissue were actually synthesized during seed formation.

CHAPTER SIX FINAL REMARKS

Plants have evolved various reproductive strategies; either producing a high number of low-investment seeds or, like lupin, fewer seeds with a higher investment per seed. Lupin seedlings exhibit a growth pattern which results in the establishment of a reliable source of water and sunlight. Initially the embryo hypocotyl expands rapidly and pushes the radicle out through the softened testa and into contact with the surrounding soil. The radicle then expands and greatly increases the surface area for contact between the soil and germinating seed. This initial growth phase occurs from 12 to 24 hours following the beginning of imbibition in the conditions used in this project.

After the water requirement has been satisfied, the stored energy is diverted towards satisfying the light requirement. A second phase of rapid elongation of the hypocotyl pushes the cotyledons upwards. Elongation slows down as light is sensed, and chloroplasts then develop in the outer cells of the hypocotyl and cotyledons. The cotyledons spread out to intercept the maximum amount of light. Once the seedling has a reliable source of water and light, further stored energy is used for growth of the epicotyl and expansion of the first true leaves, adapted primarily for photosynthesis. The seedling is then a self-sufficient plant.

A generalization drawn from other studies described in the introduction is that RNA synthesis is greatest in meristematic and early elongating tissue and that during this time, the number of ribosomes formed is sufficient to sustain subsequent cell expansion.

In the present study, it was found that the embryo hypocotyl in the seed contained 50 % of the maximum amount of RNA found in the germinating

hypocotyl. The peak rate of RNA synthetic activity for the hypocotyl as a whole, occurred at 12 hours. RNA content of whole hypocotyls doubled between 0.5 and 1.5 days. Nucleoli, the site of RNA synthesis, reached their maximum size within the first day of germination. The loss of vacuoles and granular zones accounted for the subsequent decrease in nucleolar size. At the time of maximum nucleolar size, the nucleus was often convoluted and the nuclear membrane had a high frequency of pores. The cytoplasm was densely packed with ribosomes either as polysomes or on rough e.r. When the main phase of hypocotyl elongation began, at 2 days, RNA synthesis had declined.

RNA synthesis within lupin hypocotyl cells was found to conform to the general pattern drawn from studies of other plant tissues.

APPENDIX ONE SOLUTIONS USED IN ELECTRON MICROSCOPY STUDY

Modified Karnovsky Fixative

2 % Formaldehyde

3 % Glutaraldehyde

in 0.1 M phosphate buffer pH 7.2 (see below)

To make 100 mls:

a) Heat 2 g paraformaldehyde in 80 ml water to 60-70°C

b) Slowly add 1 N NaOH dropwise until milky solution clears

c) Add buffer salts: 2.51 g $Na_2HPO_4.12H_2O$ and 0.41 g KH_2PO_4 per 100 ml

d) Add 6 ml 50 % glutaraldehyde solution

e) Make to 100 ml. Store in refrigerator.

Phosphate Buffer (0.1 M pH 7.2)

2.51 g $Na_2HPO_4.12H_2O$ and 0.41 g KH_2PO_4 in 100 ml distilled water

Glutaraldehyde Fixative

3 % glutaraldehyde in 0.025 M cacodylate buffer pH 6.8

To make 50 ml:

a) 6.25 ml of .2 M sodium cacodylate

b) 3 ml of 50 % glutaraldehyde

c) make up to 50 ml

d) adjust pH to 6.8

Osmium Tetroxide Fixative

1 % Osmium tetroxide in 0.1 M phosphate buffer pH 7.2

Durcupan ACM epoxy-resin

To make 20 ml:

10 ml component A (10.94 g)

10 ml component B (9.58 g)

0.4 ml component C

0.2 ml component D

Measure components A and B by weight into a 30 ml disposable glass vial. Mix these components by inversion of the vial then warm with hot air blower or 60°C oven and mix again. Add components C and D and once again mix thoroughly.

Uranyl Acetate Stain

Saturated uranyl acetate in 50 % ethanol.

Add uranyl acetate to 50 % ethanol until it will no longer dissolve, centrifuge and store supernatant in a brown glass vial.

Lead Citrate Stain

.025 g lead citrate

10 ml distilled water

0.1 ml 10 N NaOH (Must use small quantity of concentrated NaOH)

Shake vigourously until dissolved.

The methods listed in this section are from an unpublished electron-microscopy guide prepared by Mr A. Craig of the Electron Microscopy Unit of the D.S.I.R. Palmerston North.

APPENDIX TWO METHODS AND SOLUTIONS USED FOR ANALYSIS OF RNA

RNA Extraction Procedure

(Stewart and Letham, 1973)

Extraction Medium:

0.05 M Tris-HCl pH 7.5 buffer

0.005 M $MgCl_2$

1 % sodium laurel sulphate

To make 200 ml extraction medium:

Make 100 ml of 0.2 M Tris (2.42 g Tris to 100 ml distilled water)

Use 0.2 M HCl

Weigh 2 g sodium laurel sulphate and 0.203 g $MgCl_2$

Add 50 ml 0.2 M Tris

Add H_2O up to about 150 ml

Place on magnetic stirrer with pH meter probe in the solution

Add 0.2 M HCl until pH is 7.5. (This requires about 40 ml.)

Make up to 200 ml in a volumetric flask

Extraction Method:

a) Grind tissue in mortar and pestle with 10 times its volume of extraction medium at 0 to 4°C

b) Add 0.02 ml of DEP per ml of the above mixture

c) Incubate at 37°C for 5 minutes while stirring

d) Centrifuge mixture at 8,000 G for 5 minutes at 4°C

e) Remove the supernatant and add to it NaCl (0.1 g per ml of supernatant) and dissolve at 20°C with stirring. Incubate at 37°C for 5 minutes.

f) Centrifuge at 10,000 G for 20 min at 4°C

g) Remove supernatant and precipitate RNA with 2 volumes of 95 % ethanol

containing 0.2 M sodium acetate. Leave overnight in a deep freeze for this precipitation to occur. Centrifuge to collect precipitate of RNA and discard supernatant.

In this study the RNA was then redissolved in electrophoresis running medium as described by Penny (1971),for spectrophotometric analysis.

To make 250 ml of Electrophoresis Running Medium:

Tris 36 mM. Weigh 1.0903 g

NaH_2PO_4 30 mM. Weigh 1.1700 g

Na_2EDTA 1 mM. Weigh 0.0930 g

Glycerol 10 % by volume. 25 ml

Sodium laurel sulphate was not included because of its spectral properties.

APPENDIX THREE SOLUTIONS FOR MITOTIC ANALYSIS

Freezing Microtome Fluid

To make 100 ml:

50 ml of 10 % gelatin (5 g gelatin in 45 ml of distilled water)

7 ml glycerine jelly

50 ml distilled water

Heat gently and stir to mix

Store in refrigerator or add fungal growth inhibitor to mixture

Feulgen Reagent

(From Jensen, 1962)

Dissolve 0.5 g of basic fuchsin, 0.5 g sodium or potassium metabisulphite in 100 ml of 0.15 N HCl.

Shake mixture at intervals of 2-3 hours or until dye is converted to fuchsin-sulfurous acid.

Add 300 mg of fresh decolourizing charcoal, and shake at least 5 minutes.

Filter through hard filter paper

Store in refrigerator

Sulphur-dioxide Water

(From Jensen, 1962)

1part sodium metabisulphite

3 parts 1 N HCl

3 parts distilled water

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