GENE EXPRESSION DURING EARLY DIFFERENTIATION IN THE ROOT APEX OF *PISUM SATIVUM*

By

Elizabeth E. F. Allan

Doctor of Philosophy

University of Edinburgh

1984

I declare that this thesis has been composed by myself and that all the work herein is my own.

Elizabeth E.F.Allan

June 1984

'... mechanism and teleology are interwoven together, and we must not cleave to the one nor despise the other; for their union is rooted in the very nature of totality.'

D'Arcy Thompson

On Growth and Form, 1917

Contents Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

Page No.

CHAPTER 4: RESULTS

DEVELOPMENTAL FEATURES OF CELL DIFFERENTIATION AND MATURATION IN THE ROOT APEX OF *PISUM SATIVUM*.

Page No.

Page No.

Page No.

Page No.

ACKNOWLEDGEMENTS

I very gratefully acknowledge the advice and support of my supervisors, Dr. A. Trewavas and Dr. C.J. Leaver. I am also very grateful to Dr. R.F. Lyndon for many helpful discussions. Thanks are also due to the technical staff of the Botany Department, especially Miss H. Quinn, Mrs. P. Docking, Mr. G. Adams, and members of the departmental workshop.

I would particularly like to thank my family, and my closest friend John Duffus, for their constant support and encouragement.

This research was financed by an S.R.C. Post-graduate Studentship.

ABBREVIATIONS

A_{600}	Absorbance at 600nm wavelength
AMP	Adenosine 5'-monophosphate
AMPS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
Bis acrylamide	NN'-methylene bisacrylamide
BSA	Bovine serum albumin
Butyl PBD	2-(4'-Butyl phenyl)-5(-4''-biphenylyl)-1,3,4 oxadiazole
Ca^{2+}	Ionised calcium
CaM	Calmodulin
cAMP	Adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
Ci	Curies
2,6-DCPIP	2,6-Dichlorophenol indophenol
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylenglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
E.M.	Electron microscope
E.R.	Endoplasmic reticulum
g	Gram (s)
G	Unit of gravitational force (average)
G-6-P	Glucose-6-phosphate
G-6-Pdh	Glucose-6-phosphate dehydrogenase
GTP	Guanosine 5'-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
IEF	Isoelectric focusing
log	Logarithm
M	Molar
mA	Milliampere (s)
MES	2-(N-Morpholino) ethane-sulphonic acid
MESH	β-Mercaptoethanol

ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MWt	Molecular weight
NAD	β-Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NADP	β-Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
NEPHGE	Non-equilibrium pH gradient electrophoresis
ng	Nanogram (s)
nm	Nanometre
nM	Nanomolar
NP-40	Nonidet P40
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
pg	Picogram (s)
Pi	Inorganic phosphate
pI	Isoelectric point
pI'	Apparent isoelectric point
pm	Picomoles
pM	Picomolar
PMS	Phenazine methosulphate
PMSF	Phenylmethylsulphonylfluoride
PVPP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	NNN'-N'-tetramethyl ethylene diamine
TFP	Trifluoperazine
Tris	Tris (hydroxymethyl) amino methane
Triton-X-100	Octyl phenoxy polyethoxy ethanol
tRNA	Transfer RNA
μ	Micrometres
μg	Micrograms

µl	Microlitres
µM	Micromolar
u.v.	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume

ABSTRACT

The model proposed by Brown (1963) describing a molecular basis for cell differentiation in the root apex was investigated. This model integrates known anatomical features of development in the root apex with biochemical characteristics of development, and proposes a fundamental mechanism of regulation of cell development through progressive control of gene expression. In particular, it describes a series of enzymatic states which progresses in the same manner in all tissues, and which is regulated at the transcriptional level. This succession is proposed to control the sequential and qualitatively different physiological states of division, expansion and differentiation in the root apex.

To study this model, anatomical and biochemical features of cell differentiation were investigated. In order to examine gene expression in the root apex, tissues were dissected from serial developmental sections using length of root as a parameter of developmental time. Changes in protein composition, protein synthesis, and mRNA composition during differentiation were then analysed as follows: 1. Protein composition was examined by two-dimensional polyacrylamide gel electrophoresis of total soluble protein, and by biological activity of individual proteins. In particular, the activator-protein calmodulin, and the calmodulin-dependent and calmodulin-independent forms of NAD kinase were studied. 2. Protein synthesis was examined by two-dimensional polyacrylamide gel electrophoresis of radioactively-labelled proteins. 3. Messenger RNA composition was examined by analysis of radioactively-labelled *in vitro* translation products of extracted RNA, using two-dimensional polyacrylamide gel electrophoresis.

The pattern of cellular differentiation and the control of differentiation in the root apex appeared to be far more complex than indicated by the model of Brown; and my results were inconsistent with many aspects of the model. The major areas of disagreement include: 1. The progression of a cell through qualitatively different physiological states from a dividing state incapable of undergoing expansion and differentiation,

to a non-dividing state permitting differentiation and expansion, to a non-dividing, non-expanding, maturing state does not occur. Differentiation and expansion have already commenced at an extremely early stage in the meristem; division and expansion characteristics being dependent on cell type. Furthermore, division, expansion and maturation occur at all levels in the apical 20 millimetres of pea root. Division, expansion, and differentiation may therefore take place simultaneously in one cell, and may be interrelated rather than inherently and qualitatively different processes. This has important implications for regulation of differentiation and development.
2. Although the results were consistent with the hypothesis that differentiation is accompanied by, and regulated at least partially by, extensive changes in protein composition, no evidence for co-ordinated overall trends in enzyme activity or protein composition as described by the model was obtained; each tissue appearing to undergo a unique pattern of protein synthesis, breakdown, and final concentration. 3. Although synthesis and possibly activity of a small proportion of proteins may be regulated at the transcriptional level, no evidence for extensive transcriptional control was obtained. Regulation of gene expression during early cell differentiation and maturation in the root apex appeared to be predominantly at the post-transcriptional including post-translational level. This is in direct contrast both to the model proposed by Brown and to the currently accepted view of transcriptional control of protein composition during differentiation in the root apex.

Longitudinal zones of strictly co-ordinated and uniform physiological and metabolic activities do not appear to occur at the cellular level. Cellular development appears to proceed in a manner primarily dependent on tissue type at both the structural and metabolic levels.

CHAPTER 1 : INTRODUCTION

1. DIFFERENTIATION AND MORPHOGENESIS

A. Characteristics of differentiation and morphogenesis in plants and animals

(i) Introduction

The question of the manner and the mechanism by which a complex multicellular organism may develop from a single embryonic cell has occupied philosophers and biologists for centuries, and remains one of the major problems in Biology.

Differentiation of an organism is a highly organised co-ordinated process involving a progressive sequence of events at the molecular, cellular, tissue and organ levels. The controlled spatial and molecular divergence of cell types gives rise to an integrated functional organism. This control appears to be particularly precise in animal development, but has remarkably plastic characteristics in plant development.

In animal development the major features of differentiation are laid down in the embryo, the embryonic stage tending to be a relatively short and finite period of the life cycle during which specific regions very early become 'committed', or 'determined', to develop along specific pathways (Gardner, 1981). Morphological and biochemical differentiation, the expression of this determination, are relatively autonomous once differentiation is specified (Wolpert, 1979), and lead to divergence of cell types which generally possess a high degree of internal specialization. The adult organism then develops largely by expansion of these specialised areas. Nearly all cells in an adult animal are terminally differentiated, having undergone apparently irreversible differentiation. Only a few cell types retain a very restricted capacity for differentiation as in the formation of blood cells from a population of specialised stem cells (Metcalfe, 1981).

Differentiation and morphogenesis in plants are much more plastic, as stressed by Trewavas (1976, 1979, 1982). The mechanisms controlling differentiation may therefore not be the same as those for the regulation of animal development. Characteristics of differentiation particularly relevant to this

thesis include the observations that plants have permanent embryonic regions; that pattern formation in the embryonic regions may be readily altered; that most cells do not become highly specialised internally; and that most cells retain the capacity for redifferentiation.

In contrast to animal embryogenesis, only a very simple tissue pattern is laid out during embryogenesis from the single-celled zygote in vascular plants. Following germination, the plant develops into a far more complex organism through further cell division, expansion and differentiation (Esau, 1965). Growth, pattern formation and differentiation continue throughout the life of the plant. The two primary meristems, located at the root and the shoot apices remain permanently embryonic and constitute the main zones of growth and differentiation.

The shoot apex continually gives rise to a complicated system of new organs which may be either vegetative or reproductive, and which may alter during the life of the plant. These organs may themselves be meristematic (Lyndon, 1977, 1979); however these intercalary meristems have a determinate activity and are thus normally considered to more closely resemble organ development in animals. Tissue patterns and the pattern of organogenesis at the shoot apex are therefore rather complex.

The root is a much simpler structure, remaining cylindrical, axiate and polar, with no terminal appendages. Lateral roots do develop from the primary root, however these arise from late-forming meristematic areas which appear to originate from the pericycle, endodermis or inner cortex some distance from the apical meristem. Lateral roots then develop along a very similar pattern to the primary root (Bell and McCully, 1970).

Development of the plant embryo therefore differs from animal embryogenesis in that tissue types, tissue patterns and the number of organs develop to only a limited extent. Primary embryonic regions of the root and shoot apices, on the other hand, differ from animal embryo development in being permanently embryonic areas in which the pattern of differentiation is continually being generated, and which give rise to an indeterminate number of organs. The embryonic areas may also be readily induced to re-organise their pattern of differentiation by any

of a variety of means. Although an entire root may be regenerated directly from a very small section of the meristem (Torrey, 1955; Reinhard, 1954) or from the quiescent centre alone (Feldman and Torrey, 1976), if abnormal conditions are imposed for example by surgical interference (Wardlaw, 1965; Ball, 1948; Francis and MacLeod, 1977), by chemical interference (Barlow, 1977), or by regulating growth factors such as auxin (Torrey, 1957) or sucrose (Feldman and Torrey, 1975), the pattern will re-organise to a new stable state. Thus the tissue pattern in the apex does not appear to be predetermined, or not irreversibly determined, along a specific pathway, but rather differentiates with reference to the concurrent state of the meristem.

The capacity of plant cells for redifferentiation, even from a relatively highly specialised state, either in normal or disrupted development, is another feature of the flexibility of plant cell differentiation. For example, pericycle cells of *Zea mays* roots may undergo some degree of dedifferentiation to form the initial meristematic cells of lateral root primordia, the derivatives giving rise to cortical and procambial tissues (Bell and McCully, 1970); removal of the root cap will induce direct redifferentiation of the quiescent centre cells to root cap cells, without an intervening stage of cell division (Barlow, 1974; Barlow and Sargent, 1978); sclereids, which are normally formed only along lateral leaf margins in *Camellia japonica*, will redifferentiate from parenchyma leaf cells along leaf edges induced surgically (Foard, 1959); while secondary meristems produce new cell types from the meristematic activity of already differentiated cells (Esau, 1965b).

Many other examples of redifferentiation, usually occurring after an intervening stage of cell division, are known. This observation, in conjunction with the discovery that a complete plant may be produced through normal embryogenesis from cultures of cells obtained from a variety of tissues, has led to the concept of 'totipotency' in which any cell retaining a nucleus and some cytoplasmic structure is believed to be capable of producing an entire plant. Proof of this has not yet been obtained, and embryogenesis may not occur specifically from the

original cultured cells. However, there are many examples of *in vitro* differentiation of plant cells into different cell types (Narayanaswamy, 1977; Steward *et al.*, 1970), emphasising the more flexible nature of plant differentiation. It further indicates that genetic material is probably retained intact in most, if not all, plant cells that do not undergo selective or complete senescence as part of their normal course of differentiation; and that gene expression does not become irreversibly limited as appears largely to be the case in animal cells.

It is clear that plant cell differentiation and development are far more plastic than animal cell differentiation, and that not only do plant cells not undergo irreversible differentiation, but they may be induced to redifferentiate to a different cell type. Much of the work that has been carried out on the control of differentiation has been performed with animal cells; however, the mechanisms controlling pattern formation and subsequent cell maturation may not be the same as those for plant cells.

(ii) The root apex as an experimental system for the study of cellular differentiation

The primary root apex is a particularly attractive system for studying differentiation as it is a permanently embryonic region in which the pattern of differentiation is continually generated. Thus, it not only presents a situation different from animal cell differentiation, but provides an opportunity to investigate the nature of pattern formation as well as that of cell maturation. Unlike the shoot apex or the embryo itself, cell development is linear and continuous along the cylindrical axis of the root, and its pattern of differentiation is relatively simple. It is therefore far more amenable to study than either the shoot apex or the embryo.

Furthermore, as root tissue has been extensively employed in studies of development, many aspects of the nature of the control of, and the characteristics of, differentiation in the root apex have been investigated. Models to explain different aspects of differentiation and development in the apex have been attempted, and one in particular (Brown, 1963, 1964; Heyes and Brown, 1965) has attempted to link biochemical features with some of the known structural characteristics of development in

a model describing a molecular basis for cell differentiation and development in the apex.

The primary root apex was therefore chosen for studying gene expression during early differentiation. The aims and approach to the problem will be outlined in sections 1 B ii, 2 C ii and 2 D. The reasons for this approach will be described in the remainder of this chapter.

B. Root development

(i) Structural development of the primary root

The primary root which develops from the embryo radicle is a polar, axiate, cylindrical structure. Most tissues differentiate in concentric cylinders around the longitudinal axis, however the pattern of xylem and phloem within the central cylinder is formed of alternate radial strands.

Resumption of cell growth in the root apex may be initiated either by cell elongation (Kovalev and Obrucheva, 1977) or largely by cell division (Kovalev and Obrucheva, 1976) depending on the species. The root grows thereafter by both division and expansion, and the characteristic pattern of cell division, expansion and differentiation stabilises shortly after germination.

The main regions of growth and differentiation are largely confined to the apical three centimetres. The meristematic zone is located at the extreme apex, consisting of the meristems of the root cap and the root proper, which are separated by a region of a relatively very low rate of cell division (Goodwin and Stepka, 1945; Clowes, 1959), the 'quiescent centre' (Clowes, 1961). Cells of both the root cap and root proper are displaced away from this meristem by cell division and expansion, thus tissues grow and mature in both directions from the quiescent centre. Cells then become increasingly specialised as they progress from the meristem.

It has frequently been suggested (for example, Bünning, 1952) that differentiation may arise as a result of an initial unequal cell division. Subsequent differential development of the daughter cells occurs through unequal partitioning of

controlling cytoplasmic factors. The continuous interaction between the nucleus and cytoplasmic factors regulates subsequent development of the organism through specification of future patterns of differentiation by directioning of further unequal cell divisions and the development of cells within a lineage. A similar mechanism is envisaged to regulate embryogenesis in many animal phyla, where it appears that polarisation of cytoplasmic components prior to cytokinesis regulates subsequent gene expression according to cell lineage during early development (Davidson, 1976). This concept has been specifically applied to the root by Brown (1963) who proposes that the primary root meristem is composed of a homogeneous mass of uncommitted cells which may be induced to differentiate by means of an unequal cell division. The cell then undergoes a transition from the merismatic to the differentiating state, and subsequent differentiation occurs via a sequential and progressive interaction between the nucleus and the cytoplasm. The root apex is therefore regarded as a region of specification of cell differentiation as well as of cell maturation.

The root is traditionally regarded as being composed of successive zones of division, elongation and maturation. This concept initially arose as a result of experiments by Sachs (1873) in which the rate of displacement of markings along the root demonstrated that the maximum rate of root elongation was sub-apical. As the meristem was confined to the apex, and tissues matured basipetally, cell development was thus regarded as progressing from an uncommitted meristematic state to an expanding non-dividing state, to a maturing non-expanding non-dividing state (Brown, 1963; Chaly and Setterfield, 1975; Heyes and Brown, 1965).

The linearity and continuity of cell development, and the spatial separation of the processes of cell division, elongation and maturation has therefore led to the root becoming a popular experimental system for studying the biochemical basis of development in plant cells, and analysis of serial sections has been extensively employed to obtain data on the cellular and biochemical nature of development, and on the regulatory components of development (Brown and Broadbent, 1950; Heyes and Brown, 1965; Heyes, 1977; Loening, 1961; Chaly and Setterfield, 1975;

Torrey, 1965; Fowler and Ap Rees, 1970; Sunderland and McLeish, 1961; Navarette and Bernabeu, 1978; Steward *et al.*, 1965; Khavkin *et al.*, 1974; Sahulka, 1974).

However, in 1945, Goodwin and Stepka pointed out that the model of zonation was too simplistic, producing no quantitative information on absolute or relative rates of processes concerned, and that the nature of the transition between zones was frequently ignored. In a study of the root of *Phleum pratense*, they observed that in the apical section of the meristem of the root proper, contrary to the generally accepted view, the rate of cell division was very low. The sub-apical region of the meristem was found to have the highest rate of cell division, and also,by examining very small sections of the root, to be a region of very rapid elongation. The rate of elongation rapidly decreased at the basal end of the meristem and beyond,although in a later study they observed a second peak beyond the meristem (Goodwin and Avers, 1956). Thus, the 'zone of elongation' is an ambiguous term as, although normally applied to the elongating region excluding the meristem, cell and root elongation both occur within the meristem.

Furthermore, Goodwin and Stepka pointed out that as cells appear to differentiate at different levels, and that differentiation of some cell types clearly commences within the meristem, and others in the 'zone of elongation', that the use of the phrase 'zone of differentiation' or 'zone of maturation' is misleading. The misuse of the phrase 'zone of differentiation' or 'maturation' was also referred to by Popham (1955) in a study of the root apex of *Pisum sativum*. Popham claimed that differentiation of tissues had already occurred at the level of the transversal meristem and that differentiation of secondary tissue occurred later in root development. Differentiation and maturation, including that of secondary tissues therefore continued throughout most of the root.

More recent anatomical and quantitative observations on the relative rates and contributions of cell division and expansion to growth throughout the apex (Esau, 1965b; Goodwin and Avers, 1956; Erickson and Sax, 1956), on the different characteristics of division and expansion in cells of different tissues (Jensen, 1955b; Jensen and Kavaljian, 1958; Hejnowicz,

1959; Barlow and MacDonald, 1973; Webster and MacLeod, 1980), and on the levels of tissue differentiation (Torrey and Zobel, 1977), usually confirm and extend the observation that elongation and differentiation occur in other areas than the model indicates, and that different cell types divide and expand with different characteristics. However, the simple model of root development as consisting of zones of division, elongation and maturation is still shown in many text books (for example, Scott Russell, 1977; Cronquist, 1961). Although it appears to be more generally acknowledged that differentiation commences before cells stop elongating, a 'zone of maturation' is frequently portrayed as occurring beyond the 'zone of elongation'. Furthermore, in experimental investigation of cell development, the general nature of the model is frequently adhered to (Heyes and Brown, 1965; Brown, 1963; Oaks, 1965b; Stange, 1965; Loening, 1961; Steward, *et al.*, 1965; Chaly and Setterfield, 1975; Navarette and Bernabeu, 1978). It may also be asserted that as characteristics of any one level may be attributed to a more or less uniform population of cells (Brown, 1963; Heyes and Brown, 1965), that one may attribute the general characteristics of each zone on a cellular basis to the progress of an 'average' cell. In a consideration of controlling factors of various aspects of development, the complexity of events at the apex is similarly frequently ignored.

Experimental studies are frequently carried out on successive transverse sections or on the development of characteristics in isolated sections. In this 'zonal' method of analysis, average values per cell are obtained, and this approach has reinforced the concept that cells undergo a relatively uniform set of alterations within the apex, in particular that there is a transition from a meristematic to an expanding stage. However, this approach may be misleading.

Firstly, for example, from a series of experiments in which the characteristics of growth of isolated segments of root were examined, Brown (1963) has developed the concept that cells are initially in a meristematic state in which they do not and cannot grow or differentiate, and that they subsequently develop into a metabolic state in which they may grow and differentiate.

In these studies (Brown *et al.*, 1952; Brown and Robinson, 1954, 1955), it was observed that the apical millimetre of bean root did not increase in size at all in a 48 hour period after isolation, whereas the segment which elongated maximally, 3-4 millimetres from the tip, increased to about twice its initial length within 24 hours. The second and third millimetres from the apex both increased in length, but only after a lag period of 24-36 hours. It was therefore argued that the meristematic cells are in a metabolic state in which they cannot grow and have to develop into an enzymatic state in which growth can occur, whereas segments from the growing zone are already equipped with a metabolic system that will sustain growth (Brown and Robinson, 1955; Brown, 1963).

However, these studies do not agree with studies on elongation in the intact root in which it is clear that elongation does occur within the apical millimetre (Goodwin and Avers, 1956; Goodwin and Stepka, 1945; Erickson and Sax, 1956; Woodstock and Skoog, 1962). Furthermore, cells must clearly be displaced from the meristem by growth, otherwise the meristem would consist of cells becoming progressively smaller in size with time in the absence of compensating growth, and cells would not be displaced from the meristem. Root cap cells are also present in the apical millimetre, and as these cells elongate very close to the meristem in the intact root, this also indicates that abnormal cessation of growth has occurred.

It might also be pointed out that in examining growth in excised segments one might expect the same reasoning to be applied to the capacity of cells for division in the apex as to that for elongation. Cell division was found to be negligible in the excised segments (Robinson and Brown, 1954); however, it was not similarly concluded that cells in the meristem were equipped with an enzyme complex that did not support division.

There is much evidence to indicate that growth in isolated root sections is abnormal. For example, Brown and Sutcliffe (1950) demonstrated that the concentration of various nutrients affected growth of excised root segments. It is known (Oaks, 1965b) that amino acids from mature parts of the root are required to maintain the normal size of the amino acid pool in

the apex, and that distribution of growth substances such as auxin and cytokinins varies along the root (Barlow, 1976). Thus normal protein synthesis would be likely to be inhibited in more apical sections, and removal of the normal supply of nutrients and growth substances transported from more basal regions would also artificially alter growth. Extensive changes in metabolism are known to occur within excised plant tissues, including alteration in rate of respiration, change in the pattern of enzymes involved in carbohydrate oxidation, and in the pattern of glucose metabolism (Ap Rees, 1966). Torrey (1955, 1957) also observed an abnormal pattern of cell division in excised segments of the pea root apex and an abnormal pattern of vascular differentiation. As Barlow and MacDonald (1973) have also found that the mean cell cycle length in the apex of *Zea mays* as a whole is about twenty hours, and as cell growth compensates or more than compensates for cell division, growth must occur in the meristem normally in a 48 hour period.

Cessation of growth in the apical millimetre is therefore likely to be a result of abnormal conditions, due largely to the heterotrophic nature of the apex (Torrey, 1965) and to wounding. Differences in growth between different sections may be due to differences in the endogenous supply and requirements of various growth factors and nutrients rather than to different metabolic states. As growth in excised segments of the root apex is therefore so abnormal, it is doubtful whether reasonable conclusions about growth in the intact plant may be drawn from characteristics of their growth.

Brown also estimated cell growth by measuring mean cell volume in 400μ segments (Brown and Broadbent, 1950). However, zonal analysis of cell volume may not be adequate to detect change in cell volume from the apex of the root proper. Furthermore, although 'cell division of itself does not constitute growth' (Steeves and Sussex, 1972) in terms of an irreversible increase in size; even if no increase in volume were to occur during cell division so that the daughter cells were half the size of the original cell, it is clear that at least addition of wall, membrane and nuclear material will have

occurred, and in this context growth has certainly taken place.

Furthermore, it should be pointed out that the apical millimetre consists largely of root cap cells. The characteristics of the apical millimetre as a whole are therefore not representative of the cells originating the root proper.

Secondly, variations between different cell types emerging at the apex will not be observed by zonal analysis. This objection, however, is dismissed by Heyes and Brown (1965) who assert that variation within the root at any one level in the apical ten millimetres is minimal and may be disregarded; and by Brown (1963) who claimed that trends in a variety of characteristics such as enzyme activities or cell volume would not show such well-defined patterns of change if cell development were highly variable in different cell layers, and that it was probable that cells in all layers traversed a similar course of development.

However, there is much evidence to show that, for example, the rates and characteristics of cell division vary considerably between different tissues and with position within the meristem (Barlow and MacDonald, 1973; Webster and MacLeod, 1980). It is also clear that as tissues may be recognised by the shape and size of their cells, expansion in all planes and the addition of cytoplasmic or vacuolar materials per cell will vary between different cell types at the same distance from the apex. Thus both cell division and cell expansion are heterogeneous in the apex at any one transverse level.

A few authors who have not been satisfied with the approach that regards zones as consisting of relatively homogenous masses of cells have investigated cell size, nucleic acid content and enzyme activities in more than one area from any transverse zone, and in these cases, variations in the pattern of change between different tissues have been found (Bucknall and Sutcliffe, 1965; Sutcliffe and Sexton, 1969). Histochemical studies of the root have reinforced the observations that the pattern of enzyme development varies between tissues (Jensen, 1955a; Hall and Sexton, 1972; Sutcliffe and Sexton, 1969).

However, Heyes (1977) has claimed that these studies have simply complemented and extended studies using the serial sectioning technique, and have not invalidated conclusions

about development of an average cell drawn from the use of this method. Brown (1963) has further proposed that the pattern of change in enzyme activity is similar in all cell types at the same transverse level, and that changes occur only in the rates and in the extent to which they change in different cell types during differentiation. No evidence was obtained for this view, however, and it was based only on the observation that consistent trends were obtained during root development.

Thirdly, measurement of relatively large sections will not provide information on cytological or biochemical characteristics from the apex of the root proper. The extreme apex not only consists of dividing cells of the root proper but also of dividing, elongating and maturing root cap cells. Root cap cells also lie adjacent to the quiescent centre, so that observation of small transverse sections is subject to a similar limitation. It is also misleading to use average cell volume or cell length data for the region as a whole not only as the apical region contains root cap cells, but also as cells are continually dividing and thus constantly reducing their average size.

Finally, many authors, including Brown (1963), either due to an assumption that differentiation and division are incompatible, that differentiation is induced at a specific cell division, that embryonic cells by definition are unspecialised, or that differentiation commences concurrently with the onset of visible signs of specialisation, have equated 'meristematic' or 'embryonic' with 'undifferentiated'. In this way the concept that the root is composed of zones of undifferentiated meristematic cells which are transformed into non-dividing differentiating cells has been reinforced. However, these assumptions may not be justified.

For the purposes of investigation of characteristics at the apex, it is highly misleading to state, for example, that the apical 1.6 millimetres of pea root consists largely of 'undifferentiated meristematic tissue with few developing or elongating cells' (Loening, 1961). It is also highly misleading to state that changes observed in the subsequent sections are associated with the *onset* of differentiation or elongation (Loening, 1961). However, despite differences between cell

types along the root and the apparent overlapping of division, expansion and differentiation, the concept of cells progressing from a zone of cell division in which compensating growth may or may not occur, to a zone of rapid cell elongation in which differentiation commences, to a maturing non-dividing non-expanding state, is generally accepted. A model has been developed for the molecular basis of cell differentiation and development in the root apex on the basis of this concept (Brown, 1963, 1964), and this will be described in section 2 C i. As this model has dominated concepts on the metabolic characteristics of differentiation and development at the root apex, it was examined in further detail.

(ii) Areas of investigation

It is desirable both for the design of experiments and for the interpretation of the results to recognise the complexity at the root apex. The zonal model of cell development at the apex is an inaccurate and misleading summary, and refers to root rather than to cell development. A study of the anatomy and ultrastructure of cell development at the root apex was therefore the initial stage of investigation in this thesis. The main areas of investigation were as follows.

Firstly, as the meristematic state and differentiation in the root apex are regarded as being spatially separated and in the model proposed by Brown as possessing mutually incompatible metabolic states, I investigated the anatomical and ultrastructural characteristics of cells immediately adjacent to and distal to the quiescent centre in order to examine whether the assumption that differentiation does not progress in meristematic cells may be justified, and in order to evaluate the level at which visible signs of differentiation commence.

Secondly, again owing to the apparent spatial separation of zones of division and elongation in the root apex, and to observations on growth of isolated segments, Brown (1963) has suggested that cells in the apical meristem cannot grow, and that division and elongation are mutually exclusive in this system due to different metabolic states. As Brown based his estimates of cell growth in the intact root apex on zonal

analysis of average cell volume, I re-investigated the changes in cell shape, volume and frequency of division in different putative tissues by anatomical measurement, with reference to the apex of the root proper rather than to that of the entire root.

Finally, the levels at which various tissues differentiate have been rather arbitrarily and inconsistently defined and provide no information on the degree and type of morphological and cytological changes occurring at different levels. A study of the ultrastructural and structural differentiation of different cell types, and the level at which different characteristics appeared, was therefore carried out in order to correlate molecular development with structural development.

The term 'differentiation' has been used to refer to induction, to 'blocking out' of cell groups that will develop into a specific tissue, to the visible phase of cell specialisation, to late stages of maturation, to organ development, to embryogenesis, to germination, or to a combination of any of these. The poorly-defined use of the term is largely a semantic problem, however, in some cases the use of the term may involve assumptions about the mechanism or impose limitations on the process by which cells become different from each other. Differentiation in this text will refer to the origin and process of cell development resulting in any difference between cells at the structural, ultrastructural or biochemical level, excluding differences that do not have a developmental significance. Cells do not have to display cytologically visible differences to be regarded as in the process of differentiation, nor does the term exclude meristematic cells. Differentiation may, but does not necessarily, include a specific 'induction' phase. Maturation will refer solely to the progress of a specific developmental pathway.

2. GENE EXPRESSION DURING DIFFERENTIATION

A. Control of gene expression during differentiation

(i) Introduction

It is a common assumption that morphological and functional cell differentiation is not only accompanied by orderly changes in the pattern of protein and enzyme activities, but is a result of such changing patterns. This controlled alteration in gene expression has for some time been widely believed to occur fundamentally at the level of transcription of mRNA via a continuing and progressive interaction between controlling cytoplasmic factors and the nucleus. Determination of cells along a particular pathway may be associated with the specification of a subsequent pathway of gene activation and/or repression.

(ii) Control of gene expression in animal systems

The emphasis on transcriptional control mechanisms arose from the observations on control of gene expression in bacteria and in differentiated cells in many animal systems. In these systems there is much evidence to support the proposition that differential protein accumulation is the result of differential transcription of mRNA from a constant genome (Jacob and Monod, 1961; Davidson, 1976).

Differential transcription of mRNA due to a different number of copies of the structural gene for these proteins has been discounted in a large number of cases, as a result of cDNA hybridisation experiments to specific genes in which it has been demonstrated that the genes are present in a similar number of copies in cells in which their product is expressed in different quantities. Evidence for transcriptional regulation of gene expression also comes from cDNA hybridisation experiments to the RNA of these genes, and also from analysis of *in vitro* translation products of mRNA obtained from different cell types. In several cases, an increase in a specific protein in differentiated cells has been demonstrated to be accompanied by an accumulation of its mRNA. These include haemoglobin (Ross *et al.*, 1974; Bishop and Rosbash, 1973),

ovalbumin (McKnight *et al.*, 1975; Harris *et al.*, 1975; Palmiter, 1973, 1975), actin (Paterson *et al.*, 1974), crystallin (Berns *et al.*, 1972), fibroin (Suzuki and Suzuki, 1974), vitellogenin (Shapiro and Baker, 1977; Shapiro *et al.*, 1976), kidney androgen-regulated protein (Toole *et al.*, 1979).

However, although mRNA accumulation is frequently assumed to represent an increase in transcription of the mRNA, accumulation may also occur as a result of a decrease in rate of degradation. Furthermore, although preferential accumulation of individual mRNAs may occur, it has been pointed out by Kafatos (1972) and Trewavas (1976) that differential accumulation may arise rapidly from a non-specific increase in transcription if the mRNA species concerned have different half-lives. This may have some importance in developmental systems where mRNA species for abundant proteins such as cocoonase (Kafatos, 1972) often have a relatively very long half-life compared with other mRNA species.

However, although mRNA accumulation has been demonstrated for a number of abundant proteins in terminally differentiated cells, it is possible that many of the proteins involved in differentiation are present in very small quantities, and these may not be under a similar control mechanism. It is also possible that regulation of gene expression in differentiated cells may not resemble regulation during early stages of cell differentiation. Examples of translational control of protein synthesis are known (for example, Lodish, 1971), and post-translational control of protein might also occur during differentiation.

Transcriptional control during embryogenesis and during differentiation of individual cell types has not been investigated so extensively as in mature cells. Gel electrophoresis, however, has provided evidence that protein composition changes considerably during mammalian embryogenesis, and in at least some cases appears to be due to differential transcription (Davidson, 1976). By contrast, although the spectrum of proteins synthesised alters during early differentiation of sea urchin and amphibian embryos, this appears in the initial stages of oogenesis to be under post-transcriptional regulation

of mRNA species present in the egg at fertilisation which are provided by the diploid maternal genome. Differential gene expression occurs at least partly as a result of regionalisation of controlling factors, possibly including these mRNA species, with differential partitioning during cytokinesis. In these instances therefore, the initial stages of organogenesis do not appear to be directed by differential transcription of the embryo genome, although subsequent stages are at least partially under such control.

(iii) Control of gene expression in plant systems

a) Developmental events associated with preformed mRNA

An interesting parallel to the regulation of gene expression during early differentiation of sea urchin and amphibian eggs occurs in the early stages of morphogenesis of the single-celled alga *Acetabularia* (Kloppstech, 1982) and the water mould *Blastocladiella emersonii* (Lovett, 1982). In these organisms long-lived mRNA appears to be responsible for initial stages of morphogenesis.

There is also considerable evidence that protein synthesis during early germination of seeds is dependent on long-lived mRNA stored in the embryonic axis in the seed (Spiegel and Marcus, 1975; Peumans and Carlier, 1977; Delseny *et al.*, 1977; Hammett and Katterman, 1975). In this case, however, the mRNA species are transcribed initially from the embryo genome, not as in the case of oogenesis, from the maternal genome. It also appears that the bulk of proteins synthesised by preformed and the newly-synthesised mRNA species are very similar, and also that over 90% of preformed mRNAs are replaced within the first two hours of germination by newly-synthesised mRNAs (Marcus and Rodaway, 1982). Thus it appears that the predominantly translational control of protein synthesis during early germination is confined to a very brief period, and as the initial products are so similar it seems improbable that the preformed mRNAs are required specifically for germination or for events specific to early differentiation, but rather may be associated with rapid re-activation of growth upon imbibition.

Furthermore, the initial stages of germination are dominated by resumption of cell division and cell elongation (Kovalev and Obrucheva, 1976, 1977) and these activities do not commence immediately after imbibition. Differentiation is not observed until an even later stage of germination. As differentiation of individual cell types may therefore not commence within the first few hours of germination this further indicates that in germination preformed mRNA species may be concerned with the mobilisation of storage reserves and the activation and expansion of a preformed embryo rather than with cellular differentiation.

After the initial few hours of germination, protein composition of the embryo does change (Cumming and Lane, 1979; Caers *et al.*, 1979; Thompson and Lane, 1980; Brookes *et al.*, 1978) and this is accompanied by considerable changes in mRNA species (Brookes *et al.*, 1978). Thus it appears that alteration in transcription products of the embryo genome commences at about the time that cell differentiation commences. It is possible that new species of mRNA specific to different cell types are being synthesised in this period; however it is not known if the changing mRNA species code for proteins undergoing corresponding changes.

It therefore appears that instances in both the plant and animal kingdoms in which early development of an organism is associated with preformed mRNA, initial development may be associated with an unstable environment or an environment in which the organism is not provided directly with a source of nutrients. In development of *Blastocladiella emersonii*, and in seed activation, germination occurs when the environment is favourable. Thereafter, the environment is unpredictable, and in the case of seed germination the developing embryo has also to depend upon activation of its own reserves. In fertilisation of amphibian or sea urchin eggs, and in germination of *Acetabularia* the environment is similarly unpredictable, and may rapidly change to become very unfavourable to further development of the organism. Initial stages of development are liable to be particularly susceptible to adverse conditions, thus protein synthesis from preformed mRNA may act to allow

rapid activation and initial development of the system and thus to increase the chance of its survival. Rapid activation of enzyme activity at this post-transcriptional level in seed germination will also permit rapid breakdown of storage material for development of the embryo prior to the transition to the autotrophic stage, but may not be involved in cell differentiation. Furthermore, the range and distribution of proteins synthesised during early oogenesis in sea urchin or germination of single-celled plants may well be regulated by differential mRNA accumulation, either through regionalisation of mRNA or through prior transcriptional control although not by concurrent transcriptional control. Following the initial stages of development, however, transcription of newly formed mRNA is required for further development in all these organisms, and subsequent gene expression appears to be at least partly under concurrent transcriptional control.

Development of mammalian embryos in which transcriptional control may be demonstrated in very early stages occurs, by contrast, in a stable predictable environment in which the embryo is continually provided with nutrients.

b) Transcriptional control of gene expression in higher plants

Many plant developmental systems have been shown to be associated with specific changes in enzyme activity or protein accumulation. In some cases a concurrent change in mRNA accumulation has been indicated by nucleic acid hybridisation and by *in vitro* translation studies. Examples in which parallel changes in protein accumulation and mRNA take place include the alterations in malate synthetase, isocitrate lyase (Weir *et al.*, 1980) and the large sub-unit of ribulose bisphosphate carboxylase (Walden and Leaver, 1978) occurring in cotyledons during germination. Embryogenesis also appears to be associated with many alterations in mRNA species (Dure III, *et al.*, 1983) although for most cases it is not known if the mRNA species altering are those coding for proteins that also undergo alteration. Those proteins known to have a parallel change in accumulation of mRNA include the abundant seed storage proteins such as vicilin and legumin (Evans *et al.*, 1979). However,

although these results indicate that certain developmental events may be associated with transcriptional control, these systems are all composed of several tissues, and contain both developing and mature tissues. It is therefore not clear whether changes in gene expression may be attributed to specific events occurring during cell differentiation, or whether the changes occur in all, or in a limited number of cell types, or whether they occur as the functional response of a mature cell type in response to a particular stage of development of the organism as a whole.

Differential mRNA accumulation of proteins accumulating in individual tissues at specific stages of development has also been demonstrated. Examples include proteins such as α amylase in the aleurone layer of seeds (Higgins *et al.*, 1976); zein (Larkins *et al.*, 1976, 1983), and hordeins (Miflin *et al.*, 1983) in the endosperm of developing seeds; differential presence of the large sub-unit of ribulose bisphosphate carboxylase in bundle sheath and mesophyll cells of maize (Link *et al.*, 1978; Broglie *et al.*, 1981); the light-induced increase in the large (Walden and Leaver, 1978) and small sub-units of ribulose bisphosphate carboxylase (Sasaki *et al.*, 1981), chlorophyll a/b light harvesting protein (Apel, 1979; Apel and Kloppstech, 1978), and PAL and flavonone synthase (Schroder *et al.*, 1979). However, although involved with developmental changes these proteins are all synthesised in mature, differentiated cells, or in the case of PAL and flavonone synthase in cell cultures. Furthermore, they are abundant proteins and their control may therefore not be representative.

Thus, although the above examples concur with studies in gene expression in animal systems in which abundant tissue-specific proteins appear to be regulated at the level of transcription, and in which a variety of developmental events may also be under transcriptional regulation, no information appears to be available on gene expression during early stages of cell differentiation.

(iv) Summary

It appears in many cases both in animal systems and

in higher plants that differential mRNA accumulation may be demonstrated where abundant proteins are associated with a specific mature cell type.

In mammalian embryogenesis at least some of the early developmental changes in protein composition also reflect specific changes in mRNA composition; however, in oogenesis, in morphogenesis of certain single-celled lower plants, and in seed germination, the initial stages appear to be directed by preformed mRNA. In oogenesis this preformed mRNA is provided by the maternal genome, thus initial stages of oogenesis do not appear to be regulated directly by the embryo genome. However, in seed germination which represents re-activation of embryogenesis rather than embryogenesis itself, the stored mRNA is presumably initially transcribed from the embryo genome. The stage of dependence on preformed mRNA in this case is very brief, and it may not contribute to specific cellular differentiation. In all these instances mRNA may, however, accumulate in specific regions; or mRNA species specific to germination or early differentiation may have originally been specified at the transcriptional level. Thus germination or early differentiation in these cases may be regulated at least partially at the level of differential mRNA accumulation.

Certain stages in embryogenesis and seed germination do appear to involve concurrent transcriptional control; however it is not clear whether alteration in protein and mRNA composition may be attributed to a stage in cell differentiation of specific cell types, to a developmental stage in more than one cell type, or whether the changes are initiated in mature cells.

As control mechanisms during early developmental events in embryogenesis and in germination of embryos may therefore not resemble those in early cell differentiation during embryogenesis or during cell development from embryonic regions of the root and shoot apices for a variety of reasons, there is no indication at present as to the level at which control of gene expression in early cellular differentiation operates in higher plants.

Investigation of development of different cell types of the embryo is very difficult due to its small size. However, the root apex in early germination provides a convenient system

for analysing successive changes in gene expression occurring during early cell differentiation.

B. The biochemistry of root development: Developmental changes in protein and RNA in the root apex

As indicated in the previous section, functional activities specific to different types of mature cells may be regulated at the transcriptional level, and transcriptional control has also been demonstrated to occur in a number of developmental events although post-transcriptional controls are not excluded. However, no data are available on the regulation of early stages of differentiation in individual cell types.

The root apex provides a unique opportunity for investigation of molecular progression during early cellular differentiation. Widely-used methods of investigation include analysis of components by the serial sectioning technique as described in section 1 B i, and histochemistry.

Changes in many enzyme activities have been observed to occur as cells progress in development, either on a segment, cell or protein basis. Proteinase has been found to be very low in the meristem, and to increase towards more basal regions (Van Fleet, 1952), although as protein also increases it is not clear whether this reflects a general or a specific increase. Invertase increases almost twenty-fold per cell within the apical eight millimetres, with a ten-fold increase in specific activity. The specific activity of dipeptidase triples during the same period, whereas the specific activities of phosphatase and glycine oxidase remain about the same (Brown and Robinson, 1955; Robinson and Brown, 1952). A range of other enzymes have been indicated by histochemistry to have strongly tissue-dependent distribution, such as β glycerophosphatase and ATPase (Sutcliffe and Sexton, 1969); while a variety of enzymes as measured by *in vitro* activity also appear to have tissue-dependent variations during maturation. Peroxidase, for example, alters during root differentiation (Grison and Pilet, 1978) and displays considerable variation both in cell-free activity (Grison and Pilet, 1978) and histochemical detection between different

tissues (Jensen, 1955a; Hall and Sexton, 1972). A qualitative difference in isoenzyme pattern containing peroxidase and oxidase activities has been noted in the older regions of the root (Sahulka, 1974) indicating that different isozymes may have a developmental significance.

Respiration, which increases per cell beyond the meristem (Brown and Broadbent, 1950; Sutcliffe and Sexton, 1974), has been particularly well investigated. It appears that the glycolytic pathway and the Krebs cycle are very active in the meristem but decline towards more basal regions, whereas the activity of the pentose phosphate pathway increases (Fowler and Ap Rees, 1970; Gibbs and Beevers, 1955). The relative amounts of glucose metabolised by the two pathways therefore alters during development so that relatively more is metabolised via the pentose phosphate pathway as cells mature. Although these changes were found to be associated with corresponding alterations in enzyme activities (Gibbs and Earl, 1959) as protein content increased considerably in successive root segments, it was found that the specific activities of the enzymes of the pentose phosphate pathway in relation to protein content did not, in fact, change appreciably between segments, whereas the specific activity of enzymes involved in glycolysis decreased after five millimetres beyond the root tip in *Pisum sativum* (Fowler and Ap Rees, 1970). The change in ratio of enzyme activities is therefore due to a decrease in specific activities of the enzymes involved in the glycolytic pathway.

From further dissection of these root segments, it appears that there are differences in activities of enzymes of the pentose phosphate pathway between the stele and cortex, their activities being higher in the stele (Wong and Ap Rees, 1971). Differences in the apparent enzyme activities of the pentose phosphate pathway and the Krebs cycle as analysed by histochemical methods may also be detected between the cortex and the stele (Sutcliffe and Sexton, 1974).

It therefore appears that as protein per cell increases during early stages of growth and differentiation, this is accompanied by alteration in both total and relative enzyme activities, and that quantitative and possibly qualitative changes occur between different tissues at the same transverse

level. It should be pointed out, however, that analysis of *in vitro* activity both by assay in cell-free extracts and by histochemistry may not reflect *in vivo* activity. Limitations of substrates, pH values, compartmentation of enzyme and substrate, presence of activators or inhibitors, competition for substrates with other enzymes, may all modify enzyme activity *in vivo*. Furthermore, in only a very few cases has the presence of activators or inhibitors been investigated during *in vitro* assay (for example, Fowler and Ap Rees, 1970). Artifactual problems including enzyme solubility, drug-binding to specific tissues, lack of specificity, also limit the validity of histochemical studies.

Changes in enzyme activities either *in vivo* or *in vitro* may not reflect a corresponding change in level of the protein. However, one-dimensional polyacrylamide gel analysis has indicated that quantitative and qualitative changes in proteins also accompany root development (Steward *et al.*, 1965; Navarette and Bernabeu, 1978), so it appears that protein composition as well as enzyme activities vary throughout root development. However, one-dimensional analysis of total protein is limited in usefulness as several proteins constitute any one band, and variations in proteins present in low concentrations will be obscured. Problems may also be encountered in extractability of proteins, and in artifactual electrophoretic modifications, which may differ between different root segments. It is also not known whether changes in activities of specific enzymes are correlated with corresponding changes in the amounts of these proteins.

Several proteins have been associated with differentiation in specific cell types in other organs, and these would also be expected to vary throughout root development in association with structural differentiation. These include sieve tube exudate proteins, the major protein bands of which have electrophoretic mobilities of 15,000, 28-30,000, 59,000, 116,000 and 220,000 daltons in one-dimensional SDS-polyacrylamide gels (Weber and Kleinig, 1971). Two at least of these are highly basic (Beyenbach *et al.*, 1974), one of which comprises about 40% of the sieve tube exudate (116,000 dalton) and may be involved in

P-protein filament formation (Kleinig, 1975). Callose is also associated with phloem tissue (Cook and Stoddart, 1973), and β (1-3)glucan synthetase might therefore also be expected to increase during phloem maturation.

Lignin formation is involved in secondary wall formation of xylem, phloem fibres, and to a lesser extent endodermis, and a number of enzymes are involved in its synthesis (Hahlbrock and Grisebach, 1974). Although many of the enzymes are in common with those required for synthesis of flavonoids and cinnamate esters, induction of PAL and caffeate-O-methyl transferase activities involved in lignin synthesis does occur during secondary thickening, while the enzymes involved in synthesis of cellulose and hemicellulose also increase and those involved in pectin synthesis decrease (Northcote, 1979).

Slime, which is synthesised in abundance in outer root cap cells (Harris and Northcote, 1970), may also involve an increase in enzyme activities which are involved in its synthesis such as β (1-4) glucan synthetase.

The epidermis produces cutin in large quantities. Palmitate hydroxylase which is involved in its synthesis (Soliday and Kolattukudy, 1977) may therefore be expected to be very active in cutin-forming epidermal cells.

Change in enzyme activity, however, need not reflect change in level of protein, as post-translational controls may operate to control activity. There is evidence that total and relative amounts of proteins do appear to alter between young and older sections of the root, however, no information is available on the functions of the proteins undergoing these alterations, and alterations in protein levels have not been correlated with specific changes in enzyme activities.

The control of the changes in both enzyme activities and/or protein content in the root has been widely assumed to be attributable to transcriptional control mechanisms (Brown, 1963; Heyes, 1977; Barlow, 1982). This has arisen largely as a result of the work of Jacob and Monod (1961) on bacterial gene expression and also on the observation that both RNA and the composition of RNA alter during root development. Heyes (1960) observed that both RNA and protein content per cell increased

between the apex and more basal segments of the root, during which period the composition of RNA altered. These results indicated that relative and specific changes in protein composition might be associated with relative changes in RNA composition. However, much of the RNA examined will have consisted of rRNA, and it is probable that the bulk of the changes observed reflect changes in rRNA, and to a lesser extent tRNA. Changes in mRNA species have not been investigated.

In a more recent ultrastructural study (Chaly and Setterfield, 1975) it was noted that several components of the protein synthesising machinery and structures associated with protein synthesis altered during development of cortical tissue. Nuclear chromatin dispersion increased beyond the meristem, and later decreased beyond the zone of maximum elongation, indicating that transcriptional activity was low in the meristem and in cells that have ceased elongating. The proportion of polyribosomes and membrane-bound ribosomes to free ribosomes also altered during development, increasing beyond the meristem, rising to a peak in the 'elongation zone', decreasing thereafter, indicating that changes in translational activity may parallel changes in transcriptional activity. However, the nucleolus decreased in size before root elongation had ceased, and became predominantly fibrillar with associated chromatin in a condensed state indicating that a fall in rRNA synthesis may occur before a fall in translational activity. This was supported by the observation that (^{3}H) cytidine incorporation into rRNA fell steadily throughout development. These ultrastructural observations present indirect evidence that transcriptional and translational activity both alter throughout development of cortical cells. However, no information on qualitative and quantitative changes in individual transcripts has been obtained, and alterations in protein levels, including specific changes, may therefore reflect non-specific quantitative changes in mRNA synthesis and/or protein synthesis rather than specific alteration of either of these events.

C. Investigation of biochemical changes in the root apex

(i) A model for the molecular basis of cellular differentiation (Brown, 1963)

Although several models have been proposed for the control of pattern formation at the root apex, only one has attempted to link control and progression of molecular development with the known developmental anatomy of the root (Brown, 1963, 1964). In this model, the initial pattern-forming factors are irrelevant as differentiation is autonomous and autocatalytic following specification. Unlike most of the models concerning control of pattern formation, it is applicable to all tissues differentiating in the root apex. I therefore examined this model in further detail.

Cells in the apical meristem are regarded as being in an undifferentiated 'ground state'. Specification arises through unequal partitioning of cell components during cell division which results in different cell complements in the two daughter cells. This difference does not become apparent, and is incapable of being developed further, until cells cease division and undergo a transition from the dividing state to enter a growth and expansion phase.

Differentiation is envisaged as arising via a sequential alteration in total and relative enzyme activities throughout development, becoming evident only through growth of the cell. It was proposed that the metabolic state of cells in the meristematic phase is such that growth cannot occur. Cells at this stage possess a particular protein complex (P_1) which sustains a catalytic state (E_1) that does not permit growth. With time, the protein composition is altered through enzyme activity (e_1) to one in which growth may proceed (P_2) and which sustains a different enzymatic state (E_2). Through further enzyme activity involving a new group of enzymes (e_2) a further metabolic state (P_3) is reached where growth is arrested and another enzymatic state is sustained (E_3). All cell layers at any transverse level are proposed to undergo a common course of development in relation to timing of change in enzyme activities, activities increasing in the zone of expansion, and stabilising or decreasing after reaching a peak. Differentiation of individual cell types within this framework of change is envisaged as arising as a

result of differences only in the rate and extent of change between different tissues.

The change in enzyme activities is proposed to result from a change in amount of protein, and not from the influence of activators or inhibitors. Control of protein level is proposed to be regulated at the level of transcription by mRNA regulators produced sequentially during root development, a progressive interaction occurring between the nucleus and cytoplasm during development.

A sequential transcriptional control of enzyme protein is therefore a crucial factor in regulating development, with different tissue types arising within a general framework of change as a result of initially unequal cell complements. Thus differentiation will become apparent as cells 'grow'.

(ii) Examining the model

Evidence for this model was obtained largely from investigationof enzyme activities and growth characteristics of serial segments, or of culture of isolated serial segments. As pointed out in section 1 B i, zonal analysis provides information only on average values and does not refer to the progression of individual cell types. Brown (1963) dismissed this limitation by pointing out that the series of values obtained for a number of characteristics always show a well-defined trend, and claimed that 'it is probable that all cells in a layer traverse a similar course of development, and that the values show the change in a basic pattern of development that is common to all tissues in the growing zone'. Heyes (1977) has supported this by claiming that histochemical data and enzyme data on more detailed areas within a transverse zone have complemented rather than invalidated conclusions drawn from the use of the serial sectioning technique. This, however, is arguable, and as this has relevance not only to the characteristicsof development of protein, enzyme and mRNA species, but also to the proposal that cells in the meristem do not grow, and undergo a transition to a metabolic state in which the cells may grow, different concentric zones within serial transverse zones were examined where possible. In particular, changes in protein

composition were examined in different cell groups within a transverse section to investigate whether changes in protein composition followed the pattern proposed by Brown, changes occurring in both total and relative amounts of proteins, with increases occurring in the 'zone of expansion' and stabilising or decreasing thereafter, with differences between cell types occurring only in the degree to which and the rate at which they vary. Information on cell number, size and shape and relative proportions of different tissues were obtained from anatomical observations rather than from average values obtained from zonal analysis. Several aspects of the model that may be examined at the structural level have been described in section 1 B ii. Aspects that were examined at the biochemical level are described below.

Brown proposed that changes in enzyme activities occurred as a result of corresponding changes in their synthesis due to regulation at the level of mRNA. Changes in protein composition certainly do appear to accompany differentiation in the root apex; however, although there is some evidence to support the proposition that protein composition is regulated at the transcriptional level and protein concentration at least partially at the transcriptional level, the evidence is very indirect. It is now known that gene expression may be regulated at numerous levels other than transcription. Alteration in enzyme activity need not reflect amount, nor does amount necessarily reflect synthesis of protein, far less the regulation of the transcriptional activity of its gene.

At the time of writing, one-dimensional analysis of proteins had demonstrated that there were changes in protein composition between young and mature zones of the root, and analysis of the base composition of RNA also showed that changes in RNA composition occurred during root development. However, the protein changes were not correlated with changes in individual enzymes, and no information was available on changes in mRNA composition or mRNA sequence alteration during development. Furthermore, although enzyme activities were known to alter during development, differences in enzyme activities in different zones of the root cannot be assumed to be due to variations in the amount of enzyme as differences may reflect differences in activators

or inhibitors in those zones.

In order to make preliminary investigations of the control of gene expression during early differentiation and development in the root apex, I therefore decided to examine initially the range and degree of changes occurring in protein composition and synthesis. In order to examine whether changes in protein level might be accompanied by a corresponding alteration in synthesis, I compared the pattern of protein composition of cells by two-dimensional polyacrylamide gel analysis with the patterns of proteins being concurrently synthesised after incubation with radioactive amino acids. A preliminary investigation of transcriptional control was then carried out by *in vitro* translation of mRNA isolated from successive root segments to compare the degree of alteration in protein composition with the degree of apparent change in mRNA composition.

In order to obtain a more reliable estimation of the changes in protein levels during differentiation, and to examine the developmental significance of changes in protein composition at the root apex, I selected two proteins for further study, and examined these in further detail. In the context of the model, in which regulation is envisaged as occurring at the transcriptional level and specifically not to involve enzyme regulators, I chose to investigate two proteins that were both regulated at the post-translational level, and could in turn regulate other proteins at the post-translational level. One of these was calmodulin, a calcium-binding regulatory protein which is itself regulated post-translationally by ionised calcium fluxes, and which may also alter the distribution of this crucial regulatory ion.

There is much indirect evidence to indicate that ion distribution and concentration may be crucial in regulation of many developmental events at the root apex. Differentiation and morphogenesis at the apex involve a combination of cell division, expansion and differentiation, and calcium ions are known to regulate many aspects of these activities. The cellular responses to calcium have been found to frequently be mediated by a class of calcium-binding proteins which bind to calcium at elevated concentrations of the free ion to form active complexes. Several of these proteins have been purified

from animal cells, but only one of these, calmodulin, has been found to occur in plant tissues (Anderson and Cormier, 1978; Anderson *et al.*, 1980). Calmodulin is ubiquitous throughout the plant and animal kingdoms, and is highly conserved both structurally and functionally (Watterson *et al.*, 1980; Vanaman, 1980).

Calmodulin has been found to regulate many vital cellular processes in animal cells, regulating many key enzymes in metabolism (Klee *et al.*, 1980; Cheung, 1980; Scharff, 1981). It is also known to be involved in regulation of processes which are required by both cell expansion and cell division including the regulation of localised microtubule assembly (Schleicher *et al.*, 1982b; Kumagai and Nishida, 1979; Marcum *et al.*, 1978), tubulin kinase activity (Burke and DeLorenzo, 1982); it has been found associated with microtubule organising centres (Means and Dedman, 1980), and it is known also to be a dynamic component of the mitotic spindle (Welsh *et al.*, 1978; Anderson *et al.*, 1978; Means and Dedman, 1980). It regulates the activity of a variety of enzymes in plants including NAD kinase (Anderson and Cormier, 1978; Jarrett *et al.*, 1983), protein kinase (Hetherington and Trewavas, 1982; Salimath and Marmé, 1983), microsomal Ca^{2+}-Mg^{2+} ATPase (Dieter and Marmé, 1981b; Caldwell and Haug, 1980, 1981b; Oláh *et al.*, 1983), ATP-dependent uptake of calcium by a plasma membrane fraction (Dieter and Marmé, 1980c, 1980d), lipoxygenase and superoxide dismutase (Leshem *et al.*, 1982). Calmodulin may also regulate calcium distribution in the cell by direct binding of up to four calcium ions (Klee *et al.*, 1977; Seamon, 1980), or by activation of calcium transport pumps (Dieter and Marmé, 1981a). It has also been linked with both geotropically- and hormonally-induced events (Roux and Slocum, 1982; Chandra *et al.*, 1982; Biro *et al.*, 1982), and may therefore act as the mediator of the calcium response to hormonally-, electrically-, and geotropically-induced events.

Calmodulin may therefore either mediate or modify calcium-dependent regulatory processes in cell division and expansion at the root apex, and possibly also calcium-dependent events in differentiation. As several calmodulin-regulated activities

are present in the root apex, and these vary according to developmental position, it appeared that calmodulin would be present in the root, and that its function might vary during development. It was therefore of interest to investigate calmodulin and its potential function during differentiation in order to examine its co-ordinated and discriminating regulation of different and potentially conflicting calcium-dependent activities.

In order to investigate the significance of any alteration in concentration of calmodulin, several calmodulin-dependent activities were observed, and the activity and distribution of one calmodulin-dependent enzyme, NAD kinase, was examined. NAD kinase, like calmodulin, is regulated post-translationally. It may also, through regulation of the total and localised availability of the co-enzymesNAD and NADP, regulate the predominant metabolic pathways and exert fine control of the activities of these pathways at a post-translational level. As the NADP-requiring pentose phosphate pathway, and the NAD-requiring glycolytic pathway and Krebs cycle do in fact vary throughout the root apex, and as these pathways may regulate the channelling of carbohydrate to either provision of energy or to biosynthesis, it was of interest to investigate the potential activity of NAD kinase in the root apex in relation to the relative activities of these pathways as well as to the distribution of calmodulin.

In view of the fact that different zones of the root may contain different inhibitory or activating factors despite Brown's view to the contrary, the presence of factors interfering with the assays was investigated when estimating enzyme activity or protein content by enzyme assay or by polyacrylamide gel electrophoresis.

D. Aims and approach

(i) Aims

The aims of this project were primarily to attempt to link molecular events during early differentiation with structural and ultrastructural events at the cellular and sub-cellular

levels; and to investigate whether the epigenetic/transcription theory of differentiation in the form proposed by Brown for differentiation in the root apex may be tenable.

(ii) Approach

Although many studies have been carried out on the control of gene expression in terminally differentiated or relatively mature cells and in developmental events involving activity of several tissues in varying stages of differentiation, very little work has been carried out on early stages of cellular differentiation. This is largely as a result of the difficulties involved in handling small embryos, and in investigating different cell types during organogenesis in which complicated tissue patterns are present.

The primary root apex is a very attractive system for observing molecular and structural events involved in early differentiation. Different cell types arise either in concentric rings or in radially symmetrical patterns, while differentiation of all cell types is linear and continuous along the longitudinal axis of the root. Thus investigation of properties in serial sections will provide information on successive stages of development, while analysis of different concentric zones within these sections will provide further information on differentiation in individual tissues or groups of tissues. The root apex also provides an opportunity to study pattern formation as well as cell maturation.

The primary root apex of *Pisum sativum* was therefore initially examined at the structural and ultrastructural levels in order to be able to correlate structural and molecular differentiation and in order to be able to evaluate the significance of the processes of division and expansion for differentiation and morphogenesis.

In order to investigate the range and the type of change in protein composition and synthesis during differentiation, examination of total soluble protein from different tissues during differentiation was carried out by two-dimensional polyacrylamide gel electrophoresis. The changes in protein composition and synthesis were then compared to changes occurring

in *in vitro* translatable mRNA to obtain a preliminary indication as to the potential involvement of transcriptional control in differentiation. In order to obtain a more reliable estimation of alteration in protein content and activity, and to investigate the nature of some of the changes occurring in protein composition, two proteins, calmodulin and NAD kinase were partially purified and examined in further detail.

In order to investigate post-translational control mechanisms, the distribution and concentration of the regulatory calcium-binding protein calmodulin was studied, and its potential function during differentiation was also examined.

Reasons for the investigation of specific aspects of molecular and structural differentiation have been described in earlier sections of this chapter.

CHAPTER 2

MATERIALS AND METHODS

1. MATERIALS

A. Plant material

Pea: *Pisum sativum* (var. Feltham First) Lawson Donaldson Ltd. Seeds were stored at 4°C in the dark until required.

B. Enzymes

RNase (A), DNase, 5' nucleotidase (Grade IV), Glucose-6-phosphate dehydrogenase (Type XV), Phosphodiesterase 3':5'-cyclic nucleotide (activator deficient from bovine heart), were obtained from Sigma Chemical Company.
Creatine phosphokinase was obtained from Boehringer Mannheim.

C. Chemicals

Common reagents were obtained from BDH Chemicals Ltd., and were of 'Analar' grade unless otherwise indicated.
Ampholines pH 3-10 and pH 5-7 were obtained in a 40% sterile solution from LKB.
SDS, Acrylamide, and N,N'-methylene bisacrylamide (all specially purified for electrophoresis), were obtained from BDH Chemicals Ltd.
Agarose (Type I), D-Glucose-6-phosphate (Monosodium salt), ATP (Disodium salt, low calcium content, used for phosphodiesterase assay), ATP (Disodium salt, Grade I, used for *in vitro* translation), β-NAD (Grade III), β-NADP (Sodium salt), cAMP (Sodium salt), Spermidine, were obtained from Sigma Chemical Company.
GTP and creatine kinase were obtained from Boehringer Mannheim.
Butyl-PBD was purchased from Fisons Scientific Apparatus.
Bacto Agar was purchased from Difco Laboratories.
Nutrient Broth was purchased from Oxoid Ltd.
Araldite, Osmium tetroxide, Glutaraldehyde, (all E.M. grade) were obtained from Agar Aids.
EN^{3}HANCE was obtained from New England Nuclear.
Fluphenazine hydrochloride was the gift of E.R.Squibb and Sons Ltd.

Trifluoperazine was the gift of Smith, Kline and French Ltd.

D. Radiochemicals

L-(^{35}S) methionine, specific activity 1000-1400 Ci/mmol obtained as an aqueous solution and stored at -80°C in 1mM DTT, and L-(^{3}H) amino acid mixture, high specific activity mix were both obtained from The Radiochemical Center, Amersham U.K.
(^{3}H) trifluoperazine, specific activity 42.2μCi/mg, was the generous gift of Smith, Kline and French Ltd.

E. Miscellaneous

L-amino acids, Proteins for molecular weight markers, Calmodulin (Phosphodiesterase 3':5'-cyclic nucleotide activator from bovine heart), and bovine brain acetone powder were purchased from Sigma Chemical Company.
Nitrocellulose sheets 0.45μ pore size were obtained from Schleicher and Schüll, and 0.2μ pore size from Millipore.
Ion exchangers: DE-52 was obtained from Whatman, and DEAE Sephacel from Pharmacia Fine Chemicals.
Affinity chromatography gels: Sepharose 4B was obtained from Pharmacia Fine Chemicals, Phenothiazine-Affi gel from Biorad, Calmodulin-ultrogel from LKB.
Wheatgerm (untoasted) was obtained from General Mills.
Glass-distilled water was used throughout unless otherwise indicated.

2. METHODS

A. Cleaning and decontamination of equipment

(i) Cleaning and siliconisation of glass gel tubes for gel electrophoresis

Glass tubes were soaked for a minimum of 24 hours in chromic acid, made by dissolving 5g of potassium dichromate

in 10mls of distilled water with the subsequent addition of 90mls of concentrated sulphuric acid. The tubes were then rinsed very thoroughly with tap water followed by distilled water. After drying, the tubes were siliconised by immersion in 2% 2,4-dimethyldichlorosilane in carbon tetrachloride for 30 minutes. The siliconising fluid was left to evaporate from the tubes, which were subsequently rinsed in distilled water and then dried.

(ii) Sterilisation of equipment

Equipment was sterilised by autoclaving at 15 p.s.i. for 20 minutes where required. Sterile procedures were carried out in a sterile room maintained with ultraviolet light.

(iii) Decontamination of apparatus used for radioisotope experiments

Equipment contaminated with radioactivity was soaked for a minimum of 24 hours in 2% Decon 75 before being thoroughly washed. Scintillation fluid was removed from scintillation vials and the vials rinsed with methylated spirits before immersion in 2% Decon 75.

B. Pea seedling growth conditions

(i) Sterilisation of seeds

Seeds with discoloured or broken seed coats were discarded. The remaining seeds were surface-sterilised by soaking in absolute alcohol for 5 minutes followed by 1-2% sodium hypochlorite for 20 minutes. After extensive rinsing in sterile water over a period of 2 hours, the seeds were planted as described below.

(ii) Conditions of growth and harvesting

a) Root material

Seeds were sterilised as in section (i) and planted under sterile conditions in pie dishes of sterilised vermiculite at a depth of about 1cm. The pie dishes were covered,

then wrapped in foil to exclude light. After germination in the dark at 22-24°C for 65 hours, 80% of the roots were between 2.5 and 3.5cm long. Roots of this length were selected for study.

b) Shoot material

Seeds sterilised as in section (i) were planted in trays of perlite. If etiolated tissue was required, the seeds were then kept for 8 days in continuous darkness at 22-24°C. If light-grown shoot tissue was required, the trays were transferred to a greenhouse for 4 days after germination in the dark for 4 days at 22-24°C. The apical 15mm were harvested immediately prior to use.

(iii) Estimation of the effectiveness of the sterile growth procedure

Peas were grown under both non-sterile and sterile conditions as described in section iia. Bacterial contamination was then estimated as follows.

Single roots were gently homogenised under sterile conditions in 0.4ml of sterile water. Aliquots were transferred on to sterile nutrient agar (Bacto agar 15g/l, Nutrient broth 13g/l), and incubated at 37°C for 3 days. Bacterial colonies were counted after 3 days, and estimates of the effectiveness of the sterile procedure obtained by comparison of the number of colonies growing from roots grown under sterile and non-sterile conditions.

C. The light microscope

(i) Procedure for sectioning and staining unfixed tissue for light microscopy

Peas were grown according to the procedure in section B ii a, and root sections were cut free-hand. The sections were then stained either with toluidine blue for 30 seconds then rinsed with water, or were stained specifically for lignin with phloroglucinol and hydrochloric acid. To stain

for lignin the sections were treated with 25% HCl for 1 minute followed by 1% phloroglucinol in 95% alcohol. When the sections were almost dry, a second drop of 25% HCl was added, and the sections were examined.

(ii) Procedure for sectioning and staining fixed tissue for light microscopy

Pea roots were grown according to the procedure in section B ii a. Transverse 1mm sections were cut and fixed by immersion in 3% glutaraldehyde buffered with 0.1M sodium cacodylate pH 7.2 for 24 hours. The sections were then rinsed six times over a period of 2.5 hours in cacodylate buffer, and postfixed in 1% osmium tetroxide for 2 hours. After rinsing twice in cacodylate buffer the sections were left for 16 hours at 4°C. The sections were then dehydrated over a period of several hours with a graded alcohol series ranging from 25% ethanol to end with four changes of 100% ethanol. Following three changes in propylene oxide over 30 minutes, the sections were embedded in 1:1 araldite : propylene oxide. After allowing the propylene oxide to evaporate over one hour, an approximately equal volume of araldite was added, and the embedding mixture left for 16 hours to allow evaporation of any remaining propylene oxide. The araldite was then replaced with fresh araldite and placed under vacuum for 2 hours. After embedding again in fresh araldite the sections were incubated at 60°C for 48 hours to allow the araldite to harden.

The blocks were then trimmed and sections 1-3μm thick were cut on an LKB 4802A ultramicrotome with a glass knife. For observation under the light microscope the sections were stained for 3 minutes with 1% toluidine blue in 1% borax, rinsed with distilled water, and then with 70% ethanol. Photographs were taken using a Zeiss photomicroscope.

(iii) Cell number determination

a) Cell number determination for sections constituted of several tissues

I. Cell number was determined by the method of Brown and

Rickless (1949). Sections of root tissue were cut to 1.25mm in length by a root cutter, or were cut to smaller sections by microdissection. Five of these sections from each zone were placed in 2mls of 5% chromium trioxide for 36 hours at room temperature. Cells were then separated by rapid movement in and out of a pasteur pipette, aliquots taken, and cell number determined with the aid of a haemocytometer slide. Each estimate was obtained from 5 lots of 5 sections.

II. Cell number was estimated by combining estimates of cell numbers in individual tissues obtained as described in section b.

b) Cell number determination in individual tissues

Cell numbers in individual cell layers or tissues were estimated by counting cells in transverse and longitudinal sections under the microscope. Cell number estimates were obtained from 20-50 cells / cell layer in transverse section from 20 roots.

(iv) Determination of tissue cross-sectional area

The average cross-sectional area of a tissue at any transverse level was determined from the diameter of the outer and inner circumference of the tissue. Estimates were made under the assumption that the tissue boundaries were circular. The estimates do not allow for the area occupied by intercellular spaces, however intercellular spaces occurred only in the cortex and accounted for less than 10% of this tissue.

D. The transmission electron microscope: procedure for fixing and staining tissue

Pea roots were grown and fixed as described in section C ii. Ultrathin sections were cut with a glass knife to 60-90 nm thick on an LKB ultramicrotome, floated off on to water, stretched with chloroform vapour, and placed on a copper grid (200 mesh) for observation by the electron microscope. Staining was carried out according to Reynolds (1963) with uranyl acetate and lead citrate.

Reynolds' lead citrate was prepared as follows: 1.76g sodium citrate and 1.32g lead nitrate were dissolved in 30mls of distilled water. After addition of 8mls of 1M NaOH, the solution was taken to 50mls with distilled water.

The sections contained on grids were floated on a saturated solution of uranyl acetate in 50% ethanol and dried on filter paper. The sections were stained with Reynolds' lead citrate for 5 minutes, washed in distilled water, and dried on filter paper. The sections were then observed on an AEI EM6 electron microscope.

E. Protein estimation

(i) The Lowry method

Protein was estimated by the method of Lowry *et al.* (1951). This method relies on the reduction of the Folin reagent to a blue colour by the amino acids tyrosine, tryptophan and cysteine, and on the proportion of these amino acids in a sample relative to that in the protein used for calibration.

Lowry reagent

Alkaline copper tartrate, freshly prepared by dissolving 2% K-Na tartrate, 1% $CuSO_4$, and 2% Na_2CO_3 prepared in 0.1M NaOH, in the ratio 1:1:100.

Samples to be estimated for protein concentration were precipitated by an equal volume of 10% TCA and left for 12-24 hours at 4°C. After centrifugation at 12,000G for 10 minutes the pellet was resuspended and washed in 5% TCA twice. The pellet was then hydrolysed in a known volume of 0.1M NaOH at room temperature for 3-16 hours. Aliquots were taken and diluted to 1ml with 0.1M NaOH. 5mls of Lowry reagent were added and the solution mixed thoroughly. After waiting 10 minutes for the formation of the protein-copper complex, 0.5ml of Folin and Ciocalteus reagent diluted 1:1 with 0.1M NaOH was rapidly added while vortex-mixing. The samples were left at room temperature for 30 minutes, after which the optical

density of the blue colour which had formed was measured with a Corning Colorimeter 252 using a red (600nm) filter. Protein content was estimated relative to a calibration curve constructed with known amounts of bovine serum albumin in 0.1M NaOH. A typical calibration curve is shown in figure 2:1.

(ii) The Bradford method

Samples containing small amounts of protein were measured by the method of Bradford (1976). This is a rapid and sensitive method for the quantitation of protein. It relies on the binding of protein to Coomassie Brilliant Blue G-250 due to interaction of the dye with the $-NH^{3+}$ groups of proteins (Fazekas de St.Groth *et al.*, 1963). Binding causes conversion of the red form of the dye to the blue form, with a corresponding shift in the absorption maximum of the dye from 465 to 595 nm.

Protein reagent

100mg of Coomassie Brilliant Blue G-250 was dissolved in 50mls of 95% ethanol. After addition of 100mls of 85% (w/v) phosphoric acid, the solution was diluted to 1 litre with distilled water, and filtered. The reagent was stored at room temperature in a brown glass bottle, and was replaced every 4 weeks.

Protein samples containing 1-20µg of protein were dissolved in 5% MESH, 10mM Tris pH 7.5. Two mls of protein reagent were then added and the solution mixed by inversion. The absorption at 595 nm was measured after 5 minutes and before 1 hour of addition of the protein reagent. Protein samples of 10-100µg were treated in a similar manner, except that 5mls of protein reagent were added to the sample. The amount of protein in the sample was then determined by comparison with a calibration curve prepared using bovine serum albumin. A typical calibration curve for 0-20µg protein is shown in figure 2:2.

Figure 2:1. Lowry standard curve for protein determination

(Method based on that of Lowry *et al.* (1951)).

Fig.2:1

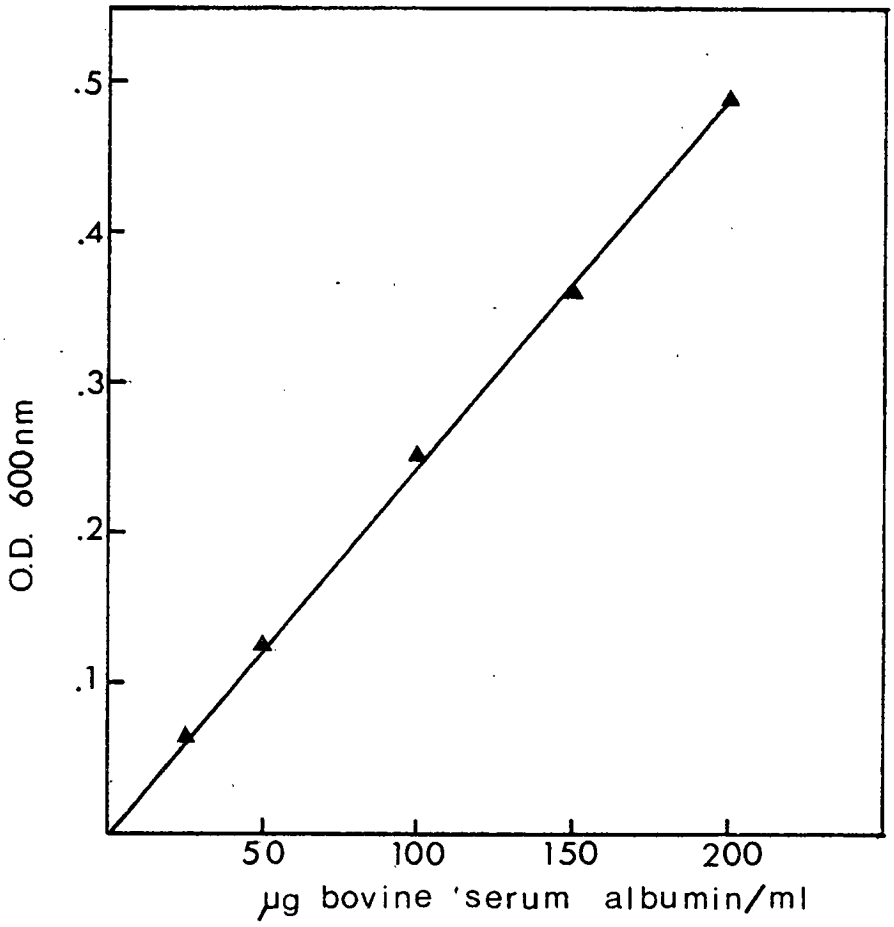
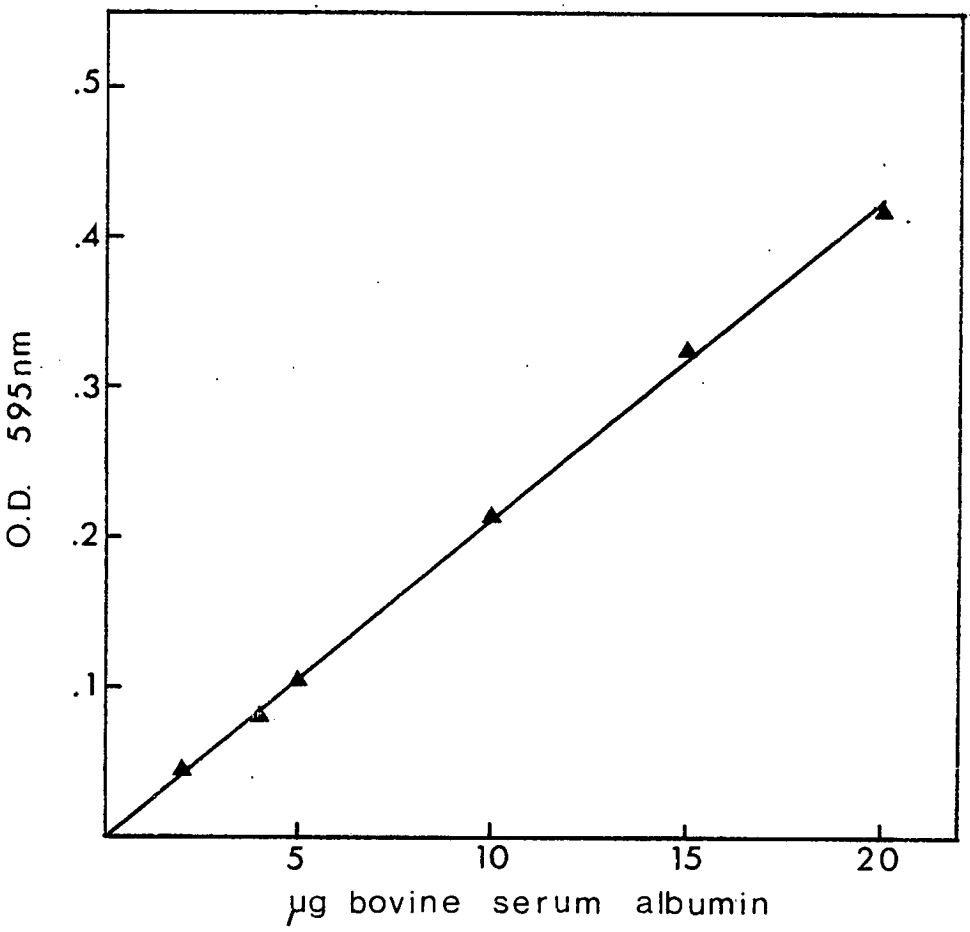


Figure 2:2. Bradford standard curve for protein determination

(Method based on that of Bradford (1976))

Fig. 2:2.



F. Incorporation of (^{35}S) methionine into protein of pea root tissue

(i) Conditions for incorporation of (^{35}S) methionine into into pea root tissue

Incorporation was carried out under sterile conditions. Pea roots were grown as described in section B ii a. Individual roots were then selected and each placed on a slanted Petri dish. 50µCi of (^{35}S) methionine contained in 10µl aqueous solution in 1mM DTT were applied to the tip of each root, and this solution rapidly moved up to the base of the root by capillary action. The lids of the Petri dishes, each containing two sheets of moist filter paper, were then placed on to the dishes to prevent the roots from drying out. The roots were left for 2 hours before being rinsed thoroughly in distilled water, and then dissected with the aid of a Nachet dissecting microscope using surgical eye blades. The sections were either used immediately, or were frozen on dry ice and stored at -80°C.

(ii) Estimation of (^{35}S) methionine incorporated into protein

Estimation of (^{35}S) methionine incorporation into protein was carried out essentially according to Mans and Novelli (1961). Root sections were homogenised in 100µl of 20mM Tris pH 7.5, and aliquots were removed for spotting on to 2.1cm discs of 3MM Whatman filter paper. After drying, the discs were immersed in 10% TCA at 4°C for at least 60 minutes to precipitate proteins and to remove RNA including amino acid-charged tRNA. After transfer to 5% TCA at 90°C for 30 minutes to remove amino acids and incomplete proteins, the discs were taken in 10% TCA at 4°C for at least 60 minutes to precipitate proteins and to remove RNA including amino acid-charged tRNA. After transfer to 5% TCA at 90°C for 30 minutes to remove amino acids and incomplete proteins the discs were taken through four changes of 5% TCA at room temperature, allowing at least 5 minutes in each change. The discs were then left in 1:1 (v/v) ether:ethanol at 37°C for 30 minutes to extract TCA and lipid, followed by two changes in ether at 35°C over 20 minutes

to remove ethanol and water. The filters were then dried, and immersed in 5 ml scintillation fluid containing 0.5% butyl-PBD in toluene. Radioactivity was then estimated using an Intertechnique SL-30 liquid scintillation counter, and counts corrected for non-specific binding of radioactivity to the filters. Estimates of the radioactivity will be referred to in the text as (^{35}S) methionine incorporation into protein.

G. Polyacrylamide gel electrophoresis of protein

(i) Separation of polypeptides by molecular weight by one-dimensional SDS-polyacrylamide gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis was carried out essentially according to Laemmli (1970).

a) Sample preparation

Samples containing protein were solubilised in 5% MESH, 62.5mM Tris pH 6.8, 2.3% SDS, 2% Ficoll. When required for identification of calmodulin, the samples were made 15mM or 10mM in EGTA or $CaCl_2$ respectively. 1µl of a saturated solution of bromophenol blue was added to each sample as a tracking dye. The samples were then heated to 100°C for two minutes, and allowed to cool.

b) Gel electrophoresis

Gel plates 15 x 16 cm were held together by greased 5mm wide teflon spacers 0.9mm thick, and clamped together with Bulldog clips.

Stock solutions were prepared and stored as follows:
N (30% stock acrylamide solution): 29.2% (w/v) acrylamide, 0.8% (w/v) bis acrylamide. The solution was filtered after dissolving the acrylamide, and was stored at 4°C. The solution was replaced after 2 weeks.
L (Separating gel buffer): 1.5M Tris-HCl pH 8.8, 0.4% SDS. Stored at 4°C and replaced after 10 days.
M (Stacking gel buffer): 0.5M Tris-HCl pH 6.8, 0.4% SDS.

Stored at 4°C and replaced after 10 days.

10% (w/v) AMPS. Stored at 4°C and replaced after 5 days.

75% (w/v) glycerol. Stored at 4°C and replaced after 1 month.

Slab gel electrophoresis buffer: 25mM Tris, 192mM glycine, 0.1% SDS. Prepared fresh each day. When required, for identification of calmodulin, the buffer was made 1.5mM in EGTA.

The plates were assembled, and a 15 ml separating gel of dimensions 15 x 11 x 0.09cm was formed between the plates, poured as a 10.5-15% exponential acrylamide gradient. The gel contained a final concentration of 0.1% SDS, 375mM Tris pH 8.8 throughout. To form the gradient, glycerol was added to a final concentration of 19% in a 15% acrylamide solution, while water was added to the lower concentration acrylamide solution to make a final concentration of 10.5% acrylamide. The solutions were degassed under vacuum, and 29µl of 10% AMPS and 10µl of TEMED were added to 15 ml of the 10.5% acrylamide solution, while 11.6µl of 10% AMPS and 4µl TEMED were added to 6 mls of the 15% acrylamide solution. The gel was then poured with the aid of a gradient maker, and overlaid with water. After polymerisation the overlay was replaced with 0.1% SDS, 375mM Tris pH 8.8.

A 4 ml stacking gel was then formed containing 0.1% SDS, 125mM Tris pH 6.8, 4.5% acrylamide. After degassing the solution under vacuum, 16µl of 10% AMPS and 5.5µl TEMED were added to the solution. The separating gel overlay was removed and the stacking gel was poured. A perspex slot former was then carefully inserted in to the gel, and the gel allowed to polymerise.

When the gel was polymerised, the slot formed was removed, and unpolymerised acrylamide removed with filter paper. The slots were filled with electrophoresis buffer. The bottom spacer of the gel was then removed, and the gel clamped inside an electrophoresis tank containing electrophoresis buffer. Air bubbles trapped between the glass plates were removed, and the top tank was filled with electrophoresis buffer. Slots were then underlaid with protein samples using a Hamilton

syringe. The electrodes were connected to the tank, (-)-ve to the top tank, (+)-ve to the bottom tank, and the gels electrophoresed at a constant current of 10mA/gel until the tracking dye reached the separating gel, after which the current was increased to 15mA/gel. The samples were electrophoresed until the tracking dye reached the bottom of the gel. The gels were then stained, destained and dried for storage.

c) Estimation of molecular weight

Lambin (1978) observed a linear relationship between the log of the molecular weight of a protein and the log of the polyacrylamide concentration that it reached during electrophoresis in a linear gradient acrylamide gel. As an exponential gradient of acrylamide effectively gives a log scale of acrylamide concentration, the log (MW) of a protein might be expected to have a linear relationship to its electrophoretic mobility in exponential gradient gels. Molecular weights of polypeptides in SDS-polyacrylamide gels were therefore estimated using this relationship by employing the method of Weber and Osborn (1969) which was developed for gels of a single acrylamide concentration.

Marker proteins of known molecular weights were electrophoresed adjacent to protein samples. A calibration curve was then constructed of the log (MW) of these marker proteins plotted against their electrophoretic mobilities. The apparent molecular weights of polypeptides of unknown molecular weight were then estimated by comparison with these marker proteins. A calibration curve is shown in figure 2:3.

(ii) Separation of proteins by one-dimensional non-denaturing polyacrylamide gel electrophoresis

Proteins were separated by one-dimensional polyacrylamide gel electrophoresis by modifying the procedure of Laemmli (1970) for denaturing gels.

a) Sample preparation

Samples were solubilised in 62.5mM Tris pH 6.8, 5% MESH,

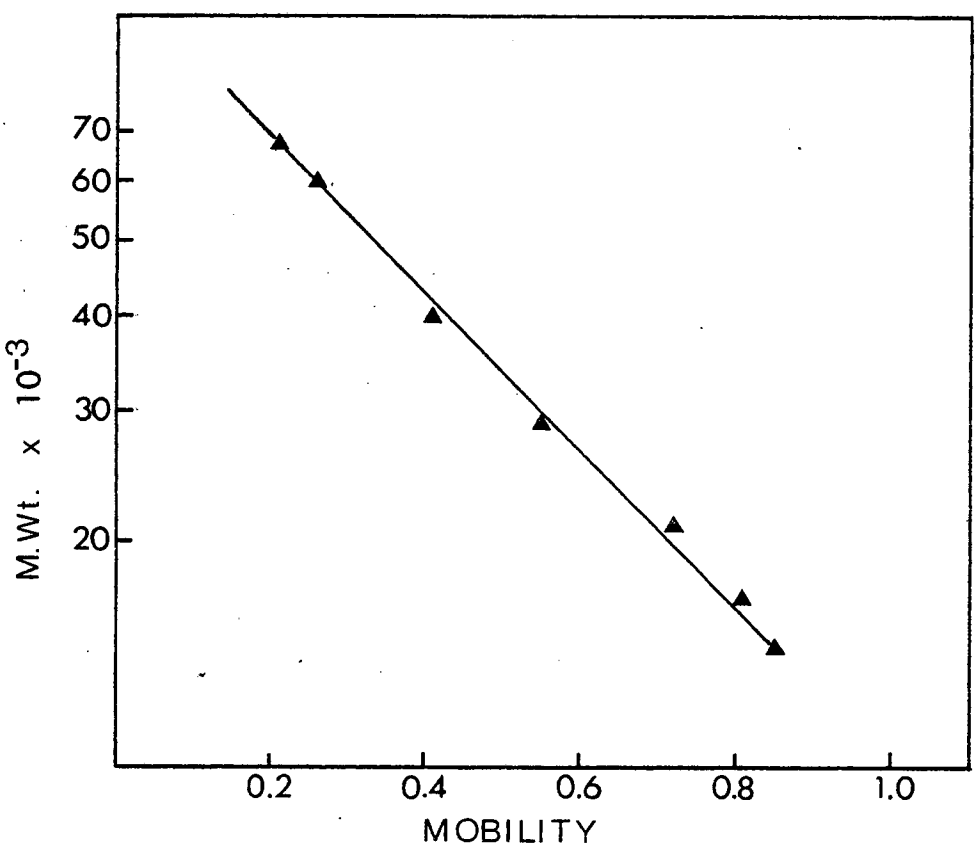
Figure 2.3 Calibration Curve for the Estimation of Molecular Weight of Polypeptides Separated by SDS-Polyacrylamide Gel Electrophoresis

Proteins were electrophoresed on a 10.5-15% exponential acrylamide gradient gel as described in section G i. The electrophoretic mobilities of the molecular weight markers were plotted against the log of their known molecular weights as described by Weber and Osborn (1969)

Molecular Weight Markers:

Bovine serum albumin	:68,000
Catalase	:60,000
Aldolase	:40,000
Carbonic anhydrase	:29,000
Soybean trypsin inhibitor	:21,000
Myoglobin	:17,000
Lysozyme	:14,300

Fig. 2:3.



2% Ficoll. When required, the buffer was made 15mM, 10mM or 6μM with respect to EGTA, $CaCl_2$ and calmodulin respectively. 1μl of a saturated solution of bromophenol blue was added to each sample.

b) Gel electrophoresis

Gels were prepared exactly according to the procedure in section i b, with the exception that SDS was omitted from all the buffers.

(iii) Separation of polypeptides on the basis of charge and molecular weight by two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis was carried out by modifying the procedure of O'Farrell (1975) as discussed in chapter 3 section 1.

a) First dimension

Proteins were separated in the first dimension on the basis of charge. Stock solutions were prepared as follows:

D (Stock 30% acrylamide for first dimension gels): 28.38% acrylamide, 1.62% bis acrylamide. Filtered before storage at 4°C. Replaced after 2 weeks.

A (Sample buffer, and second gel overlay): 9.5M urea, 2% (w/v) NP-40, 5% (v/v) MESH,ampholines pH 3-10. Stored at -20°C.

H (First gel overlay): 8M urea. Stored at -20°C.

K (Sample overlay): 9M urea, 1% (v/v) ampholines pH 3-10. Stored at -20°C.

O (Equilibration buffer; agarose gel buffer): 10% (w/v) glycerol, 5% (v/v) MESH, 2.3% (w/v) SDS, 62.5mM Tris pH 6.8. Stored at 4°C. Replaced after 1 month.

Electrophoresis buffer (upper reservoir): 0.2% H_2SO_4. Freshly prepared.

Electrophoresis buffer (lower reservoir): 0.5% ethanolamine. Freshly prepared.

I. Isoelectric focusing: sample preparation

Method 1: Sonication in RNase.

Sonication in RNase was carried out essentially according to O'Farrell (1975).

Root sections were placed in sonication buffer 10mM Tris pH 7.4, 5mM $MgCl_2$, 50µg/RNase/ml, with the addition of 1% MESH, and were sonicated at 4°C for two 3-second bursts. DNase was added to make a final concentration of 50µg/ml and the sample was left on ice for 5-10 minutes. The sample was then made 9M in urea, and an equal volume of buffer A was added before electrophoresis.

Method 2: Solubilisation in urea.

Root sections were homogenised in lysis buffer of the same composition as that of O'Farrell (1975) using ampholines of a greater pH range of pH 3-10 (buffer A).

Method 3: Solubilisation in SDS.

Solubilisation of proteins by SDS was carried out by a modification of the method of Ames and Nikaido (1976).

Root sections were homogenised gently in buffer containing 0.2% SDS, 5% MESH, 50mM Tris pH 7.4, 0.5mM $MgCl_2$, and were then sonicated for two 3-second bursts. DNase was added to a final concentration of 50µg/ml and the sample left on ice for 5-10 minutes. The sample was then heated to 100°C for 1 minute, and after cooling was made 9M in urea. Two volumes of buffer A containing 8% NP-40 were added before electrophoresis.

II. Non-equilibrium pH gradient electrophoresis: sample preparation

NEPHGE was carried out by a modification of the procedure of O'Farrell *et al.* (1977). Root sections were homogenised as for method 2 in section I above. The homogenisation buffer A was made 15mM in EGTA or 10mM in $CaCl_2$ when required for identification of calmodulin.

III. pH gradient estimation

0.5cm sections of a blank gel were cut immediately after electrophoresis, and placed in 2 mls of distilled water or 9.2M urea. After 2 hours the solutions were mixed and the pH read on an E.I.L. pH meter. The pH gradient values in the text are those obtained using water, unless otherwise specified.

IV. Gel electrophoresis

Siliconised glass tubes, 15cm long with an inside diameter of 2.5mm, were sealed at one end with parafilm. The gel solution containing 9.2M urea, 2% ampholines pH 3-10, 2% NP-40, 4% acrylamide, was degassed under vacuum and 20µl 10% AMPS and 14µl TEMED were added/10ml solution. The gel tubes were loaded with the acrylamide solution and overlaid with buffer H. After polymerisation, the overlay was replaced with buffer A.

To load the samples, buffer A was removed, and the sample loaded and overlaid first with buffer K and then with 0.2% H_2SO_4. Gels were run from the acidic (anodic) end unless otherwise indicated. Electrophoresis was at 400V constant voltage for 16 hours for isoelectric focusing and 5 hours for NEPHGE resulting in pH gradients of 4.6-9.3 and 3.8-9 respectively. After electrophoresis the gels were equilibrated with buffer O for 1.5 hours, frozen on dry ice, and stored at -80°C.

b) Second dimension

Proteins were separated in the second dimension on the basis of molecular weight in discontinuous SDS slab gels as adapted by O'Farrell (1975) for two-dimensional electrophoresis.

The procedure was essentially the same as that for one-dimensional SDS-polyacrylamide gel electrophoresis as described in section G i b. In order to load the first dimension gel, however, a straight-edged perspex spacer was inserted to a depth of about 2mm in to the stacking gel in place of the slot former. After polymerisation, the unpolymerised acrylamide

was removed and the freshly thawed first dimension gel was secured on top of the stacking gel with the aid of a 1% agarose gel dissolved in buffer O. After the agarose gel had solidified, the bottom spacer of the gel was removed and the plates were clamped to an electrophoresis tank filled with electrophoresis buffer as described before. After removal of air bubbles trapped between the glass plates, the upper tank was filled with electrophoresis buffer and 0.1 ml of a saturated solution of the tracking dye bromophenol blue was added to the upper tank. Marker proteins in sample buffer containing 2% Ficoll were pipetted into a small slot made in the agarose gel adjacent to the first dimension gel. The electrodes were then connected and the gel electrophoresed as for one-dimensional SDS gels.

(iv) Visualisation of proteins in polyacrylamide gels

a) Fixing and staining protein with Coomassie brilliant blue

Gels were fixed and stained by shaking gently for 30 minutes in 0.1% Coomassie brilliant blue R250 in 50% methanol, 7% acetic acid. Destaining was achieved by three 1 hour washes in 25% ethanol, 8% acetic acid. The gels were stored if necessary in 5% methanol, 7% acetic acid.

b) Fixing and silver staining of protein

Fixing and silver staining of protein was carried out by a modification of the method of Switzer *et al.* (1979). Stock solutions were prepared as below:

Paraformaldehyde: 850 mls of distilled water were heated to 55°C. 40 g of paraformaldehyde were dissolved in this, followed by 14.3 g of sodium cacodylate. The pH was adjusted to pH 7.2-7.4 with HCl, and the volume was brought to 1 litre with water.

Cupric-silver reagent: For 1 gel, 1.75g of silver nitrate were dissolved in 50 mls of distilled water, and 0.75 mls of 0.5% cupric nitrate was then added. A solution of 2 ml pyridine and 4 mls of absolute ethanol was then added to the cupric-silver mixture while stirring.

Diammine reagent: For 1 gel, 15 mls of 10.4% silver nitrate were added to 11.1 mls of a sodium-ammonium hydroxide solution (made from mixing 100 mls of 0.36% NaOH with 45 mls concentrated ammonium hydroxide). After disappearance of a transient brownish precipitate, 27.5 mls of 20% ethanol were added. This solution was used within 5 minutes of preparation.

Reducer I: 100 mls of ethanol, 6 mls 1% citric acid and 2.5 mls 3.7% formaldehyde were brought to 1 litre with distilled water.

Reducer II: 100 mls ethanol, 5 mls 1% citric acid and 5 mls 3.7% formaldehyde were brought to 1 litre with distilled water.

Reducer III: Solution A: 37 g NaCl and 37 g cupric sulphate were dissolved in 850 mls of distilled water. Concentrated ammonium hydroxide was added until the precipitate that had first formed was completely dissolved. The volume was adjusted to 1 litre with distilled water.
Solution B: 436 g of sodium thiosulphate were dissolved in 1 litre water.
Solution A and B were mixed in equal volumes and diluted if necessary. The solution was used within an hour, before loss of the greenish-blue colour.

Proteins in the gel were fixed for 30 minutes in 50% methanol, 12% acetic acid followed by two hours in 10% ethanol, 5% acetic acid. After three 5 minute washes in 10% ethanol, staining was carried out in a fume cupboard as follows:

The gel was shaken in paraformaldehyde solution for 30 minutes. After three 5 minute washes in 10% ethanol, the gel was transferred to the cupric-silver solution for at least 30 minutes, and then to the diammine solution for 10 minutes. The gel was then quickly transferred to reducer I with constant agitation for two minutes for the reductive liberation of metallic silver. The solution was replaced by reducer II, and after optimal staining was achieved after about 3-5 minutes, the development was rapidly stopped by shaking briefly in 5% acetic acid followed by extensive rinsing in distilled water. Background staining was reduced by reducer III diluted to an appropriate concentration.

c) Drying gels

Gels were rinsed in water and laid on to a flexible plastic film, and a square of Whatman 3MM chromatography paper was laid on top of the gel. This assembly was placed, chromatography paper down, on a plastic support connected to a water vacuum. The vacuum was sealed and the gel dried under vacuum and mild heat at about 60°C for 1.5 hours.

d) Autoradiography of radioactively labelled proteins

Dried gels were exposed to Kodak Regulix BB5 film at room temperature for 6 weeks in complete darkness. The film was developed under a red safe-light in Ilford Phen-X developer, and fixed in Ilford IF-23 fixer.

e) Fluorography of radioactively labelled protein

Gels with ^{3}H as the source of radioactivity, or with a low level of ^{35}S, were fluorographed to increase the sensitivity of detection of the radioisotope (Bonner and Laskey, 1974; Laskey and Mills, 1975). This was carried out by shaking in 50 mls of EN^{3}HANCE for 1 hour followed by precipitation of the fluorescent material in the gel by washing in distilled water for 1 hour. The gel was then dried and exposed to Kodak X-Omat XH 1 or Du Pont Cronex-4 film that had been pre-exposed as described by Laskey and Mills (1975). Pre-exposure was carried out by flashing the film for 0.001 second with a Sun Pak GT 32 flash gun covered by an Ilford S902 filter which was covered with a Whatman Grade 1 filter paper to diffuse the light. The flash gun was held at a height of about 20cm from the film to produce an O.D. at 540nm about 0.15 units higher than the background fog level of untreated film. The dried gel was placed on the preflashed side of the film and exposed for 2 weeks to 2 months at -80°C in the dark. Developing was carried out in complete darkness or under a red safe-light with Ilford Phen-X developer, and fixed in Ilford IF-23 fixer.

(v) Identification of calmodulin following Western transfer of protein from polyacrylamide gels onto nitrocellulose paper

a) Procedure for Western transfer of protein from SDS and non-denaturing gels

Western transfer of protein from polyacrylamide gels was carried out according to Towbin *et al.* (1979) with minor modifications. Gels were shaken in transfer buffer containing 0.1% SDS, 192 mM glycine, 25mM Tris, 20% methanol for 30 minutes, and then placed on a sheet of 0.2µ pore size nitrocellulose which was lying on a sheet of Whatman 3MM chromatography paper. Air bubbles were removed, and a sheet of chromatography paper was placed on top of the nitrocellulose. Chromatography paper and nitrocellulose were pre-soaked in transfer buffer. The assembly was sandwiched first between two scouring pads, then between two plastic grids, and was then inserted into a tank filled with the transfer buffer. Electrophoresis was for two hours at 56V, 400mA, with the nitrocellulose on the anode side of the gel.

When required, staining was carried out according to Burnette (1981) by agitation of the transfer in 0.2% Coomassie brilliant blue R 250, 40% methanol, 10% acetic acid for 5 minutes. Rapid destaining was achieved by 90% methanol, 2% acetic acid, and the transfer was then rinsed thoroughly with water to remove the destaining solution.

The nitrocellulose sheets were not normally stained before incubation in (^{3}H) trifluoperazine. In this case the transfers were either stored in sterile distilled water, or were incubated in (^{3}H) trifluoperazine immediately after rinsing in sterile water.

b) Binding of (^{3}H) trifluoperazine to calmodulin immobilised on nitrocellulose paper

Nitrocellulose strips were either incubated directly with (^{3}H) trifluoperazine, or were pre-incubated with bovine serum albumin to block additional binding sites on the nitrocellulose paper. Incubation in 3% bovine serum albumin, 0.9% NaCl, 20mM Tris pH 7.4 was carried out at room temperature for 1.5 hours, followed by 5 changes of 0.9% NaCl, 20mM Tris pH 7.4 for 30 minutes. The nitrocellulose sheets were then incubated in

10μM (^{3}H) trifluoperazine, 1mM $MgCl_2$, 10mM Tris pH 7, and either 2mM EGTA or 1mM $CaCl_2$. Samples were shaken for varying lengths of time at room temperature, either in complete darkness, or in ultraviolet light at 254nm with irradiation at a distance of 10cm from the surface of the transfer. The transfers were then washed in the dark in six changes of 10mM Tris pH 7, 1 mM $MgCl_2$, and either 1mM $CaCl_2$ or 2mM EGTA, for a period of 30 minutes.

The strips were then dried by blotting between filter paper, sprayed with EN^{3}HANCE, and fluorographed.

H. Purification of proteins

(i) Calmodulin

a) Preparation of a phenothiazine affinity column

A phenothiazine affinity column was prepared by the method of Charbonneau and Cormier (1979) using the bisoxirane coupling method of Sundberg and Porath (1974). Deionised distilled water was used throughout.

40 mls of Sepharose 4B were washed with water on a sintered glass funnel with a glass fibre filter, then suction-dried under vacuum. 25 g of this suction-dried material were added to 25 mls of 0.6M NaOH containing 75 mg of sodium borohydride, then 25 mls of diglycidyl ether added and the mixture stirred gently at room temperature for 8 hours. The gel was washed with 2 l of water to stop the reaction, and was then suction-dried under vacuum.

The gel was immediately activated with 40 mls of 0.1M sodium carbonate buffer pH 11 containing 101 mg fluphenazine hydrochloride. In all subsequent stages the gel was kept in the dark, as phenothiazines are light-sensitive. The gel was incubated at 70°C for 48 hours, stirring gently. Coupling was terminated by washing on a glass funnel with 500 mls of acetone to remove unreacted fluphenazine. Excess oxirane groups were removed by washing with 250 mls of absolute ethanol for 30 minutes. The coupled gel was stored in the dark at 4°C in 1mM NaN_3.

b) Purification of bovine brain calmodulin

Calmodulin was purified from bovine brain by a slight modification of the method of Caldwell and Haug (1981a). All procedures were carried out at 4°C.

7.8 g of bovine brain acetone powder were homogenised in 160 mls of buffer containing 50 mM Tris pH 7, 2mM EDTA, 0.25 mM PMSF, 1mM MESH, for a total of two minutes at high speed in an atomix. The homogenate was stirred for 1 hour and then centrifuged for 30 minutes at 7,500G. The supernatant was filtered through glass wool, and solid ammonium sulphate was added over a period of 1 hour to 55% saturation. The pH was readjusted to pH 7, and the solution stirred for 1 hour. After centrifugation at 12,500G for 30 minutes, the pellet was discarded and the supernatant adjusted to pH 4 with 1M H_2SO_4 containing ammonium sulphate at 55% saturation. The solution was stirred for two hours and then centrifuged at 12,500G for 30 minutes. The pellet was resuspended in buffer A (20mM MES-NaOH pH 7, 300mM NaCl, 1mM MESH) + 5mM $CaCl_2$ and dialysed against this buffer for 12 hours.

The dialysate was clarified by centrifugation at 100,000G for 1 hour, and the supernatant loaded on to a 10 ml fluphenazine-Sepharose 4B affinity column prepared as described in section a) which had been pre-equilibrated in buffer A containing 1mM $CaCl_2$. To remove unbound material, the column was washed extensively with this buffer at a flow rate of 50 ml/hour until the A_{235} was less than 0.005.

Calmodulin was then eluted with 10 mM EGTA replacing 1mM $CaCl_2$ in the buffer. Fractions showing an increase in A_{235} were pooled and taken to 80% saturation with ammonium sulphate. After two days the solution was centrifuged at 12,000G for 30 minutes, and the pellet resuspended in 2 mls of 20mM Tris pH 7.4 and dialysed against this buffer for 12 hours. The dialysate, which contained homogeneous calmodulin by the criterion of two-dimensional polyacrylamide gel electrophoresis, was then frozen in liquid nitrogen and stored at -20°C. Calmodulin was stable at this temperature for at least one year.

c) Purification of calmodulin from *Pisum sativum*

I. Partial purification of calmodulin from pea root tissue

Peas were grown as described in section B ii a. Several hundred 1.25mm sections from each zone to be examined were homogenised in 10 volumes of buffer containing 1M KCl, 2mM $MgCl_2$, 1mM EGTA, 50 mM Tris pH 7.4, 2.5% (w/v) PVPP. After centrifugation for 30 minutes at 20,000G, the supernatant was taken to 60% saturation with ammonium sulphate over a period of 1 hour, and the pH adjusted to pH 4 with 1M H_2SO_4 containing ammonium sulphate at 60% saturation. After stirring for 12 hours, the solution was centrifuged at 12,000G for 30 minutes, and the pellet resuspended in and dialysed against 20mM Tris pH 8, 1mM EGTA for a further 12 hours. The sample was heated to 100°C for 2 minutes to destroy heat-labile proteins, and the denatured protein was removed by centrifugation at 12,000G for 30 minutes. The supernatant was then dialysed against 20mM Tris pH 8 for 12 hours to remove the EGTA, and the dialysate tested for presence of calmodulin by NAD kinase activation. The samples were then frozen in liquid nitrogen and stored at -20°C. Calmodulin was purified almost to homogeneity by this procedure by the criterion of two-dimensional polyacrylamide gel electrophoresis.

II. Purification of calmodulin from *in vitro* translation products

Calmodulin was isolated from *in vitro* translation products by phenothiazine affinity chromatography using phenothiazine-affi gel obtained from Biorad.

400µl of *in vitro* translation products containing the equivalent of 3,000,000 c.p.m. of TCA-precipitable material were diluted 1:1 with 20mM Tris pH 8, 1mM $MgCl_2$, 1mM MESH. $CaCl_2$ was added to a final concentration of 1mM, and the sample was immediately applied to a 1.5 ml column of phenothiazine-affi gel that had been equilibrated with the same buffer containing 1mM $CaCl_2$. Chromatography was carried out in the dark at an initial flow rate of 5ml/hour. A further 2 mls of buffer were passed through the column, and the eluate was re-

applied to the column. Material binding in a non-specific manner was then eluted with 50 column volumes of buffer containing 1mM $CaCl_2$ and 300mM NaCl at a flow rate of 10 mls/hour until no radioactivity could be detected by liquid scintillation counting. Material bound in a calcium-dependent manner was then eluted with 3mM EGTA replacing $CaCl_2$ in the buffer. The EGTA eluate was assayed for the presence of calmodulin by NAD kinase activation and was stored at -20°C.

(ii) NAD kinase

a) Partial purification of NAD kinase activity from pea shoot tissue by the method of Anderson *et al.*

NAD kinase was partially purified from pea shoots by the method of Anderson *et al.* (1980). Calmodulin-deficient, calmodulin-dependent NAD kinase activity was separated from calmodulin-independent NAD kinase activity, and the calmodulin-dependent form was used to assay calmodulin.

Peas were grown as described in section B ii b. All extraction procedures were carried out at 4°C.

50 g of pea shoot tissue were homogenised in 200 mls of buffer A (1M KCl, 50mM Tris pH 7.4, 2mM $MgCl_2$, 1mM EGTA, 0.5mM PMSF) containing 2.5% PVPP. KCl was added to a final concentration of 1M, and the homogenate was filtered through two layers of cheesecloth. The filtrate was centrifuged at 12,000G for 30 minutes, and the pellet was discarded. The supernatant was diluted 1:1 with distilled water, and loaded onto an anion exchange column of 100 mls DEAE-Sephacel equilibrated with the same buffer diluted 1:1. The extract was eluted at a flow rate of 1.5 mls/minute with buffer A diluted 1:1, and ammonium sulphate was immediately added to 50% saturation over a period of 1 hour. After equilibration for ½ hour the solution was centrifuged at 12,000G for 20 minutes, and the pellet re-suspended in a minimum volume of buffer A diluted 1:9 with distilled water. After dialysis against this buffer for 12 hours, the dialysate was centrifuged at 24,000G for 1 hour to clarify the solution. The supernatant was then loaded on to a second DEAE-Sephacel column equilibrated with buffer A diluted 1:9,

and was eluted with this buffer.

Ten ml fractions eluting from the column were tested for both NAD kinase activity and dependence of NAD kinase activity on calmodulin as described in section I ii. All calmodulin-dependent NAD kinase activity was found to elute at this salt concentration, and all NAD kinase activity detected was completely dependent on calmodulin. The fraction containing the highest NAD kinase activity when fully activated with bovine brain calmodulin was used for assay of calmodulin according to the procedure in section I iii. The enzyme activity was found to have a half-life of 20 hours, and was therefore prepared fresh for each batch of calmodulin assays.

When the absorbance at 280nm of the column eluate had returned to baseline value, protein more strongly bound to this column was eluted with buffer A diluted 1:9, with the addition of KCl to a final concentration of 0.4M. Fractions were again tested for NAD kinase activity and dependence on calmodulin. All the calmodulin-independent activity was found to elute at this salt concentration. No calmodulin-dependent activity was detected as addition of calmodulin did not increase activity, and activity could not be reduced by trifluoperazine, an inhibitor of calmodulin.

b) Extraction of NAD kinase activity from pea shoot tissue by the method of Anderson and Cormier

NAD kinase was extracted from pea shoot tissue by the method of Anderson and Cormier (1978).

Peas were grown according to the procedure in section B ii b. All extraction procedures were carried out at 4°C.

100 g of pea shoot tissue were homogenised in 300 mls of 25 mM Tris pH 8, 2% (w/v) PVPP. After straining through two layers of cheesecloth, the homogenate was centrifuged at 12,000G for 30 minutes, and the supernatant taken to 50% saturation with ammonium sulphate over a period of 1 hour. After equilibration for a further ½ hour, the solution was centrifuged at 12,000G for 20 minutes, and the pellet resuspended in and dialysed against 25 ml Tris pH 8, 0.1mM EGTA, 0.1mM NaCl to an electroconductivity equivalent to 0.1M NaCl.

The dialysate was passed through a 100 ml DE-52 anion exchange column equilibrated with 25mM Tris pH 8, 0.1mM EGTA, and was eluted with this buffer and a linear gradient of 0-0.6M NaCl. The electroconductivity of each fraction was tested to estimate the salt concentration of the fraction, and all fractions were assayed for NAD kinase activity and dependence on calmodulin. NAD kinase activity extracted by this method was found to be entirely independent of calmodulin.

c) Extraction of NAD kinase activity from pea root tissue

Pea roots were grown according to the procedure in section B ii a. All extraction procedures were carried out at 4°C.

One to two hundred 1.25mm sections weighing approximately 200 mg in total were cut from different zones of pea root. The tissues were then homogenised in 20 volumes of 1M KCl, 1mM EGTA, 50mM Tris pH 7.4, 2mM $MgCl_2$, 0.5mM PMSF, 2.5% (w/v) PVPP and centrifuged at 25,000G for 30 minutes. The supernatants were dialysed against 50mM Tris pH 7.4, 2mM $MgCl_2$, 1mM EGTA, 0.2M KCl, 0.5mM PMSF for 2 hours. NAD kinase activity was assayed 4 hours after harvesting.

d) Separation of calmodulin-dependent and calmodulin-independent NAD kinase activities by calmodulin-affinity chromatography

Peas were grown as described in section B ii, and were subjected to calmodulin-affinity chromatography using calmodulin-ultrogel obtained from LKB. 10 g of tissue were homogenised in 3 volumes of the buffer of Anderson *et al.* (1980): 1M KCl, 1mM EGTA, 50mM Tris pH 7.4, 2mM $MgCl_2$, 2.5% (w/v) PVPP, 0.5mM PMSF. The homogenate was centrifuged at 25,000G for 30 minutes to remove cell debris and PVPP, and the supernatant dialysed against several changes of 1mM EGTA, 50mM Tris pH 7.4, 2mM $MgCl_2$ for two hours to remove the KCl. $CaCl_2$ was added in excess over EGTA to produce a final concentration of 3mM $CaCl_2$, and the dialysate was immediately applied to a 2 ml calmodulin-ultrogel column equilibrated with 3mM $CaCl_2$, 50mM Tris pH 7.4, 2mM $MgCl_2$ at a rate of 3 ml/

hour. Unbound material was eluted with this buffer + 400mM KCl for 16 hours. When the A_{280} values had reached 0.050, material binding in the presence of calcium was eluted with EGTA added in excess of $CaCl_2$ to a final concentration of 5mM EGTA. Fractions of an elevated A_{280} were pooled. The original dialysate, the EGTA eluate, the void volume and the calcium eluate immediately prior to EGTA elution were all retained for NAD kinase assay and gel electrophoresis.

(iii) Calmodulin-binding proteins

a) Purification of calmodulin-binding proteins from shoot tissue

Peas were grown as in section B ii b. 16 g of shoot tissue were homogenised and treated as in section H ii d above for the purification of NAD kinase, with the exception that the flow rate of material through the column was 12 mls/hour. When the A_{280} values of the calcium eluate had reached the baseline value, proteins binding to the column in a calcium-dependent manner were eluted with 5mM EGTA final concentration in the buffer. Eluted proteins were examined by two-dimensional polyacrylamide gel electrophoresis.

b) Purification of calmodulin-binding proteins from root tissue

Peas were grown as described in section B ii a. Seven hundred 10mm sections of pea root were cut, and homogenised and treated as in section H ii d above. When the A_{280} of the calcium eluate had reached 0.050, calmodulin-binding proteins were eluted with a final concentration of 5mM EGTA in the buffer. Eluted proteins were examined by two-dimensional polyacrylamide gel electrophoresis.

I. Enzyme and protein assays

(i) Estimation of calmodulin-dependent bovine brain phosphodiesterase activity

Phosphodiesterase activity was estimated essentially

according to the method of Cheung (1971). This was a three stage procedure involving incubation of phosphodiesterase with cAMP to form 5'-AMP, followed by incubation of this 5'-AMP with 5'-nucleotidase to form adenosine and inorganic phosphate. The inorganic phosphate released was then measured by the method of Fiske and Subbarow (1925) by conversion of phosphomolybdate followed by reduction to a phosphomolybdenum complex that was blue in the presence of sulfite. The optical density of the blue colour, which could be measured spectrophotometrically by absorbance at 660nm ± 40nm, was proportional to the phosphate concentration.

I. 0.005 units of activator-deficient phosphodiesterase were preincubated for 10 minutes at 30°C in buffer either in the presence or absence of calmodulin. The reaction was initiated by the addition of cAMP. Final concentrations of reactants in a total of 500µl were 2mM cAMP, 40mM Tris pH 8, 0.1mM $MnCl_2$, and 1mM $CaCl_2$ or 2mM EGTA. Incubation was carried out for 10 minutes at 30°C, and was terminated by heating to 100°C for 3 minutes. The solution was then allowed to equilibrate at 30°C for 20 minutes.

II. 1 unit of 5'-nucleotidase from *Crotalus Atrox* snake venom in 50µl distilled water was added to the solution, and incubated for 10 minutes at 30°C. The reaction was terminated by 50µl of 55% TCA.

III. 750µl of distilled water was then added to the solution followed by 150µl of 1.25% ammonium molybdate in 2.5N H_2SO_4. Denatured protein was removed by centrifugation for 10 minutes at 12,000G in an MSE Micro Centaur microcentrifuge. Inorganic phosphate in the supernatant was then measured by addition of 50µl of Fiske and Subbarow reducing reagent. The blue colour was allowed to develop for 10 minutes, and the absorbance at 660nm was read within the following 10 minutes.

Inorganic phosphate released from the reaction was then estimated by comparison with a calibration curve prepared as described below. Estimates of phosphodiesterase activity were adjusted for controls incubated without cAMP.

A calibration curve of phosphate concentration was prepared by dissolving different concentrations of KH_2PO_4 in 1.35 ml of distilled water. 0.15 ml of 1.25% ammonium molybdate in 2.5N H_2SO_4 were then added, followed by 50µl of Fiske and Subbarow reagent. After leaving the solution for 10 minutes for the blue phosphomolybdenum complex to form, the absorbance at 660nm was read within the following 10 minutes. A typical calibration curve is shown in figure 2:4.

1 unit of phosphodiesterase activity is defined as the amount required to hydrolyse 1 µmole of 3':5'-cAMP to 5'-AMP/ minute under the conditions specified. 1 unit of calmodulin with respect to phosphodiesterase activation was that amount required to produce half-maximal activation of 0.005 units phosphodiesterase.

(ii) Estimation of NAD kinase activity

NAD kinase activity was assayed by a modification of the method of Muto and Miyachi (1977). This was a two stage procedure. The initial stage involved incubation of NAD kinase with NAD and ATP to form NADP. In the subsequent stage, NADP formed in the first stage was assayed by NADP-dependent G-6-Pdh catalysed conversion of G-6-P to form 6-phospho-gluconate and $NADPH_2$. The $NADPH_2$ formed reduced PMS, which in turn reduced 2,6-DCPIP. Reduction of 2,6-DCPIP was measured spectrophotometrically as a decrease in absorbance at 600nm.

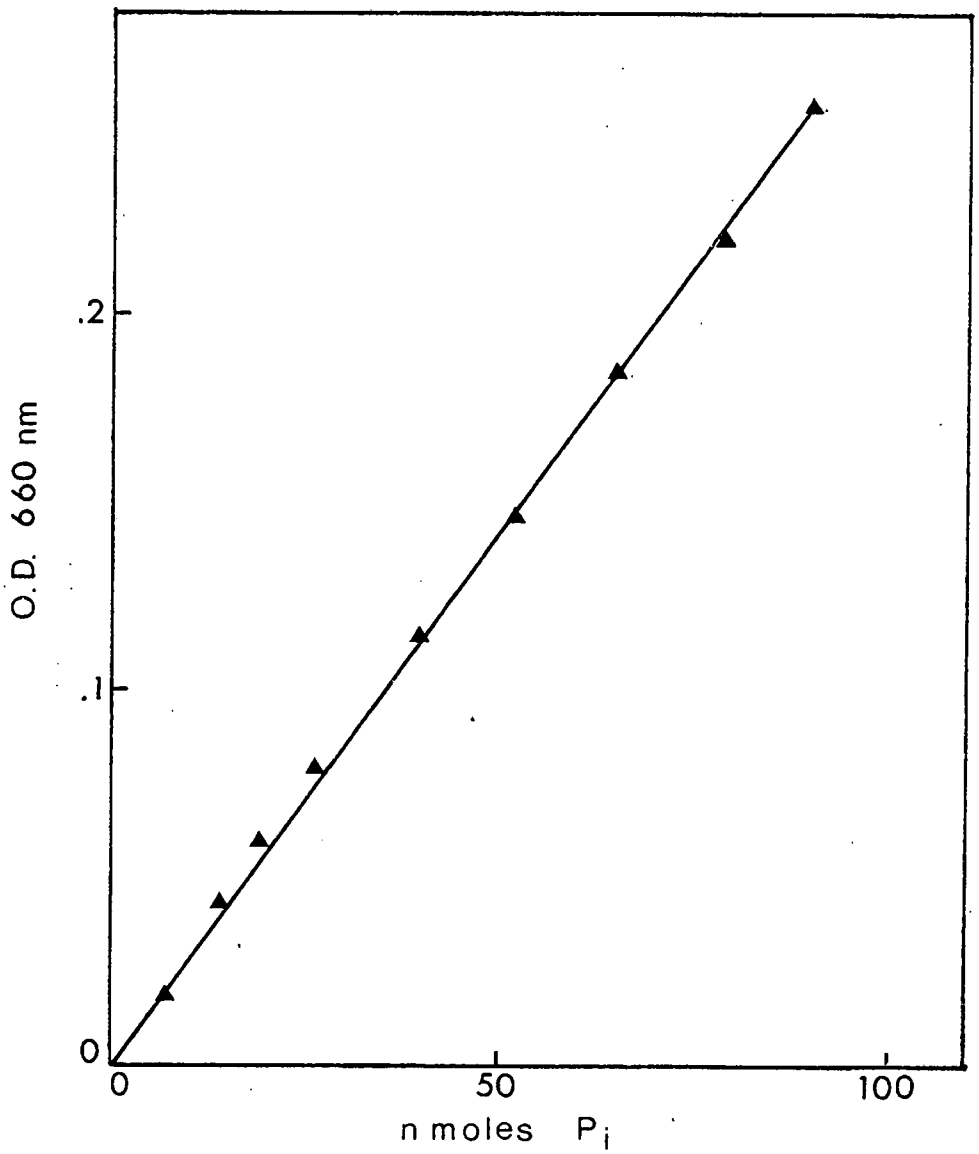
I. NAD kinase samples prepared as in section H ii were incubated at 37°C in a final volume of 0.5 ml containing 3mM ATP, 10mM $MgCl_2$, 40mM Tris pH 8, 1mM $CaCl_2$, 2mM EDTA, 2mM NAD. The reaction was initiated by addition of NAD, and incubation was carried out for 30 minutes. The reaction was terminated by addition of 0.1 ml of 1M HCl, then neutralised with 0.1 ml of 1M NaOH. The denatured protein was removed by centrifugation for 3 minutes in a microcentrifuge, and the clear supernatant used to determine concentration of NADP.

II. The supernatant was preincubated in a cuvette in a Pye-Unicam SP8-100 ultraviolet spectrophotometer with the addition of 2mM G-6-P, 30µg 2,6-DCPIP and 20µg PMS. After 1-3 minutes,

Figure 2:4. Standard curve for the determination of inorganic phosphorus

(Method based on that of Fiske and Subbarow (1925))

Fig. 2 : 4.



when the absorbance at 600nm gave a constant reading, the reaction was initiated by addition of 1 unit of NADP-dependent G-6-Pdh. The rate of decrease in absorbance at 600nm was recorded, and the initial velocity over the first two minutes was used to estimate NADP concentration.

NADP formed was estimated with the aid of a calibration curve prepared using authentic NADP in place of NAD kinase in the initial reaction. Three calibration curves were prepared for different ranges of NADP from 1 pmole to 10nmoles. A calibration curve for the middle range of 100-1000 pmoles is shown in figure 2:5. NAD kinase activity was estimated after adjusting the values obtained for controls incubated without NAD.

Calmodulin-independent activity was estimated as the activity of NAD kinase when incubated in the presence of 0.1mM TFP or 2mM EGTA.

Calmodulin-dependent activity was estimated by subtraction of the value obtained for calmodulin-independent activity from that obtained for the activity of NAD kinase when fully activated with calmodulin.

1 unit of NAD kinase activity is defined as the amount required to convert 1 µmole of NAD to NADP/minute when fully activated. The specific activity of calmodulin-dependent NAD kinase activity 30 hours after harvest was 0.05 units/mg protein.

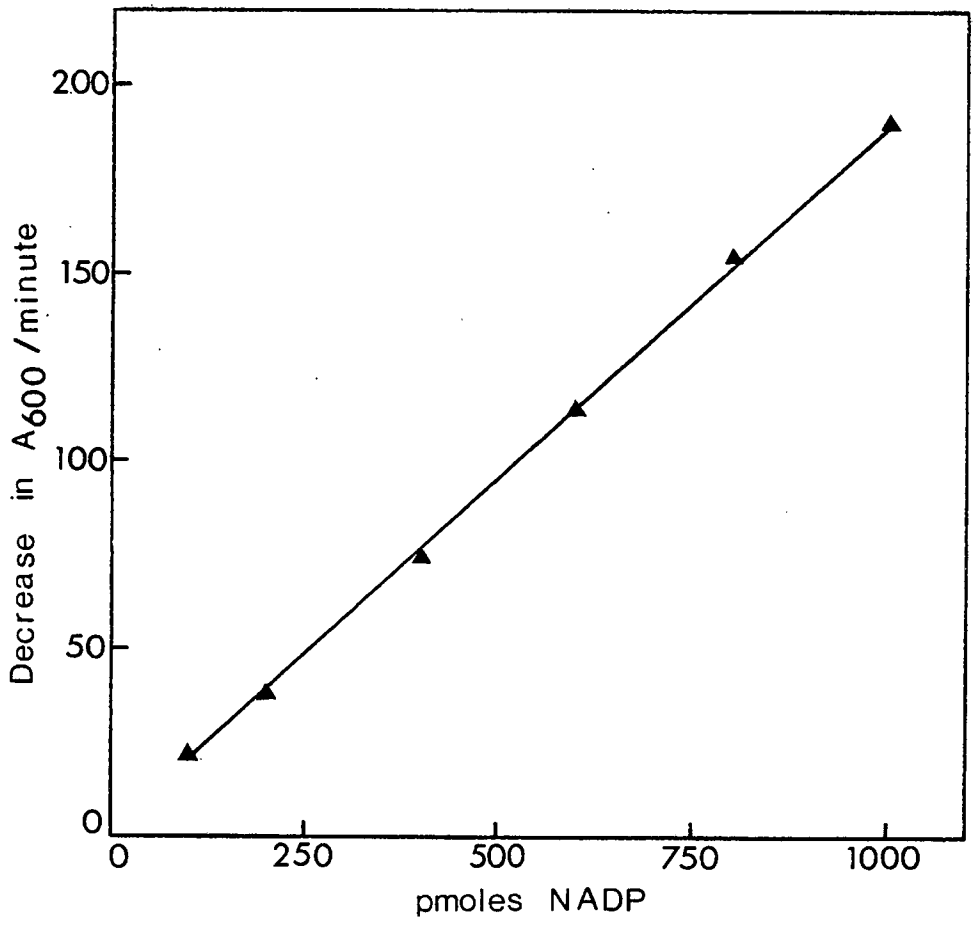
(iii) Estimation of calmodulin by activation of calmodulin-dependent NAD kinase

The concentration of calmodulin in a sample was estimated by activation of 50µl (typically 0.005 units) of calmodulin-deficient, calmodulin-dependent NAD kinase obtained by the method of Anderson *et al.* (1980) as described in section H ii a. NAD kinase assays were carried out by the modified method of Muto and Miyachi (1977) as described in section I ii. The concentration of calmodulin in a sample was estimated by comparison with activation by a known amount of bovine brain calmodulin obtained by the method of Caldwell and Haug (1981a). A typical calibration curve using 50µl (0.005 units) of NAD

Figure 2.5 NADP Standard Curve for the Estimation of NAD Kinase Activity

NADP was assayed by the modified procedure of Muto and Miyachi (1977) for the estimation of NAD kinase activity as described in section I ii. NAD Kinase was replaced by NADP in the assay.

Fig. 2:5.


200
150
100
50
0
Decrease in A_{600} /minute
0
250
500
750
1000
pmoles NADP

kinase is shown in figure 2:6. Estimations of calmodulin were made using the linear section of the curve. The enzyme activity had a half-life of 20 hours, therefore all NAD kinase incubations were carried out immediately after partial purification of the NAD kinase, 27-30 hours after harvesting; and the enzyme was recalibrated with bovine brain calmodulin for each experiment.

Controls were carried out on all calmodulin samples to check for NAD kinase activity and to estimate the presence of NAD, NADP, $NADPH_2$, or any material capable of reducing 2,6-DCPIP in the absence of G-6-Pdh activity. NAD, ATP, NAD kinase,calmodulin and G-6-Pdh were all found to be necessary for a decrease in absorbance at 600nm, indicating that the decrease in absorbance was entirely due to calmodulin-dependent NAD-kinase activity during incubation, that calmodulin samples did not contain NAD kinase activity, and that endogenous pyridine nucleotides did not interfere with the assay. Adjustment of the values obtained for NAD kinase activity was therefore not required.

1 unit of calmodulin is defined as that amount required to produce half-maximal activation of 0.005 units of NAD kinase.

J. *In vitro* translation of RNA from *Pisum sativum* in a wheatgerm-S30 cell-free protein synthesising system

(i) Extraction of RNA

a) Extraction of total nucleic acids

Nucleic acids were extracted from pea root tissue by a modification of the method of Leaver and Ingle (1971).

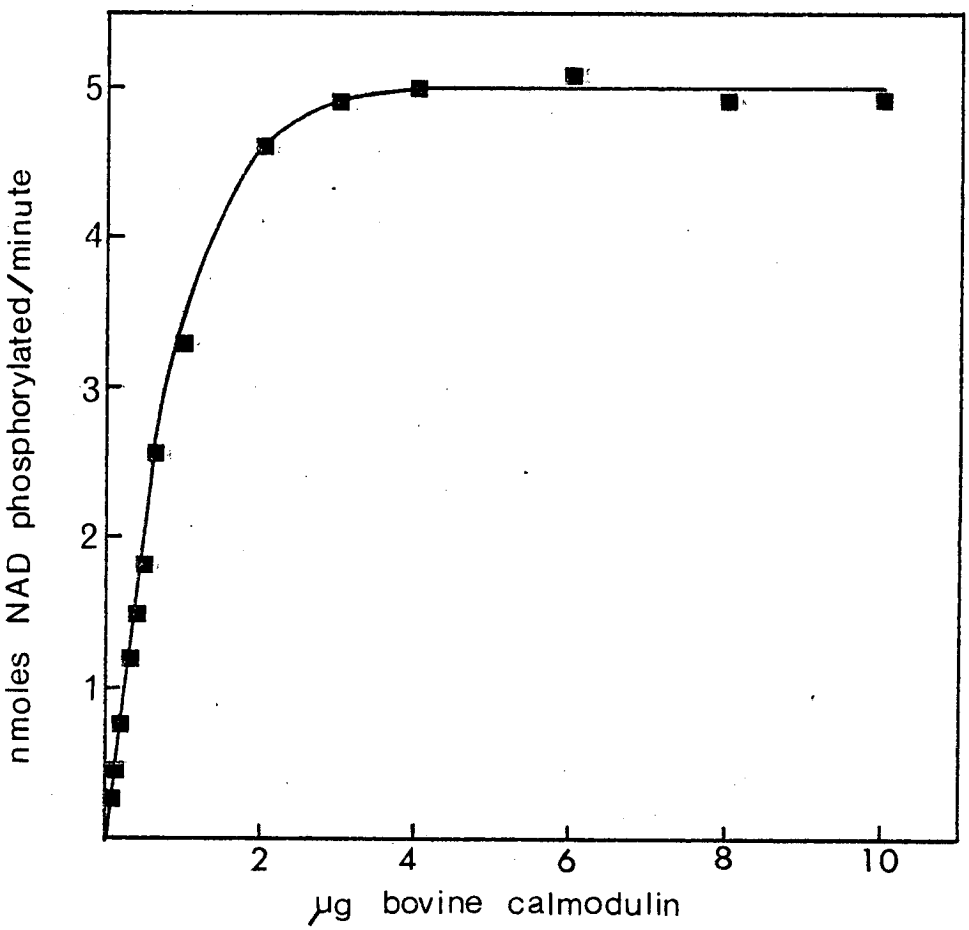
Pea root sections were cut and rapidly frozen in liquid nitrogen or on dry ice, ,and were stored at -80°C before use. Procedures for the extraction of RNA were carried out at 4°C.

Root tissue was ground with a mortar and pestle in two-five volumes of detergent-containing medium (6% paraamino-salicyclic acid, 1% tri-iso propylnaphthalenesulfonic acid, sodium salt, 0.1M Tris-HCl pH 8.5) to inactivate nucleases

Figure 2.6 NAD Kinase Standard Curve for the Estimation of Calmodulin

Calmodulin-dependent NAD kinase was assayed by a modification of the procedure of Muto and Miyachi (1977) as described in section I iii. Activation of NAD kinase was achieved using bovine brain calmodulin purified by the procedure of Caldwell and Haug (1981a).

Fig. 2:6.


nmoles NAD phosphorylated/minute
1
2
3
4
5
2
4
6
8
10
µg bovine calmodulin

and to dissolve nucleoprotein complexes. Proteins were then removed from the homogenate by phenol extraction in one volume of phenol/cresol solution consisting of phenol containing 10% redistilled m-cresol and 0.1% 8-hydroxyquinoline, and saturated with 0.1M Tris-HCl pH 8.5. After thorough mixing, the samples were centrifuged in siliconised centrifuge tubes at 2,000G for 15 minutes, and the aqueous phase re-extracted twice with approximately equal volumes of phenol/cresol solution or until no denatured protein could be observed at the interface. Nucleic acids were then precipitated from the aqueous phase with 0.1 volumes of 3M potassium acetate pH 6 followed by 2-2.5 volumes of absolute ethanol at -20°C. After precipitation for 2 hours at -40°C, nucleic acids were collected by centrifugation at 2,000G for 15 minutes. The pellet was washed twice with 80% ethanol in the presence of 0.25mM ammonium acetate to maintain nucleic acid in salt form. The pellet was finally dried in a vacuum desiccator.

The pellet was resuspended in sterile distilled water and stored at -20°C. A rough estimate of the concentration of nucleic acids was obtained by measuring the absorbance at 260nm of a 1:200 dilution under the assumption that an optical density reading of 1 was equivalent to 40µg of nucleic acids/ml. An estimate of the purity of nucleic acids was obtained by measuring the A_{260}/A_{280} ratio which was between 1.9 and 2 for all samples.

b) Extraction of mRNA and rRNA from nucleic acids for *in vitro* translation

DNA, tRNA, 5S rRNA and carbohydrate were removed from total nucleic acids by solubilising the salt-ethanol nucleic acid precipitate of section a) above in 2-3M potassium acetate pH 7. After precipitation on ice for at least 24 hours, the mRNA and rRNA were collected by centrifugation at 10,500G for 30 minutes. The pellet was resuspended in 70% ethanol containing 0.25mM ammonium acetate and centrifuged at 6,000G for 15 minutes.

The pellet was dried in a vacuum desiccator, and the RNA dissolved in sterile distilled water. A rough estimate of

concentration was obtained by measuring the absorbance of the sample at 260nm under the assumption that an optical density reading of 1 was equivalent to 30µgRNA/ml. The RNA was stored at a concentration of approximately 2.5mg/ml at -20°C, and was used to direct *in vitro* protein synthesis in a wheatgerm S30 cell-free protein synthesising system. Immediately before translation the RNA was heated to 65°C for 10-15 minutes, then rapidly cooled on ice.

(ii) Preparation of a wheatgerm-S30 extract

The preparation of a wheatgerm extract for *in vitro* translation was adapted from the study of Marcu and Dudock (1974). All operations were carried out at 4°C.

5 g of wheatgerm were ground to a fine powder with the aid of 5 g of powdered glass in a pestle and mortar. 13 mls of buffer A containing 20mM HEPES-KOH pH 7.6, 5mM Mg acetate, 120mM KCl, 6mM MESH were added, and the mixture ground to a paste. This homogenate was centrifuged at 30,000G for 15 minutes. The lipid fraction was discarded and the supernatant collected and passed through a Sephadex G25 Course column that had been equilibrated with buffer A. Material was then eluted with buffer A at a flow rate of 3ml/minute, and the A_{260} of the eluted fractions was monitored. Fractions with an optical density greater than 100 were pooled and centrifuged at 30,000G for 15 minutes. The supernatant was dialysed for 18 hours against at least 3 changes of buffer A with 1mM DTT replacing MESH. The dialysate was then centrifuged at 30,000G for 15 minutes and the supernatant dispensed into 200µl aliquots, frozen in liquid nitrogen, and stored at -80°C.

(iii) Conditions of incorporation of (^{3}H) amino acids by the wheatgerm-S30 cell-free translation system

A wheatgerm cell-free protein synthesising system was prepared by combining wheatgerm extract prepared as described in section ii above, with a mixture of (^{3}H) amino acids and a reaction mix containing a buffered source of energy, cations, tRNA, creatine phosphokinase, and amino acids (minus (^{3}H) amino acids). Protein synthesis was directed by RNA obtained

as in section J i, normally in a final volume of 50µl. Components of the reaction mix were stored as follows:

20mM ATP, 1mM GTP, 160mM creatine phosphate were dissolved in 20mM HEPES-KOH pH 7.6.
Creatine phosphokinase was stored as a 0.5% (w/v) solution in 50% glycerol.
0.5mM of each of a mixture of amino acids were stored in 10mM HEPES-KOH pH 7.5.
400mM HEPES-KOH pH 7.6, 5mM spermidine, 2.1mM potassium acetate, 25mM magnesium acetate.
200mM DTT.
10mg tRNA/ml.
All components were stored at -20°C. All equipment in contact with the incubations was sterilised, and all solutions were prepared with sterile water, to minimise nuclease activity.

For a 50µl incubation, the cell-free protein synthesising system was prepared by pipetting 7µg RNA into an Eppendorf centrifuge tube in a total of 25µl of sterile distilled water. 10µl of the reaction mix were then added to produce final concentrations of 28mM HEPES-KOH pH 7.6, 130mM potassium acetate, 2.25mM magnesium acetate, 0.25mM spermidine, 1mM ATP, 50µM GTP, 8mM creatine phosphate, 5µg creatine phosphokinase, 2mM DTT, 10µg tRNA, 0.025mM amino acids. 5 µCi of (^{3}H) amino acids contained in 5µl were then added. The reaction was initiated by addition of 10µl of freshly-thawed wheatgerm extract, and rapid vortex-mixing. Incubation was carried out at 25°C for 90 minutes. The reaction was terminated either by transferring to ice, or by addition of electrophoresis buffer for polyacrylamide gel electrophoresis. A time course of incorporation was followed by removal of 5µl aliquots at different time intervals and pipetting on to Whatman 3MM filter paper for estimation of TCA-precipitable (^{3}H) as described in section iv.

(iv) Estimation of (^{3}H) amino acid incorporation into protein

Incorporation of (^{3}H) amino acids into protein during *in vitro* translation was estimated by the procedure of Mans and Novelli (1961). 5µl aliquots were removed from the *in*

vitro translation incubations and pipetted onto 2.1cm discs of Whatman 3MM filter paper. The discs were then carried through the procedure described in section F ii. This procedure measures (^{3}H) incorporation into material precipitable by hot 5% TCA, and will be referred to as incorporation of (^{3}H) amino acids into proteins or into TCA-precipitable material.

(v) Analysis of *in vitro* translation products by polyacrylamide gel electrophoresis

Samples from *in vitro* translation were either solubilised directly in sample buffer for one-dimensional SDS or for two-dimensional NEPHGE-SDS polyacrylamide gel electrophoresis as described in section G; or they were first precipitated with 4 volumes of acetone. After precipitation of the proteins in acetone at -20°C, the protein was pelleted by centrifugation at 12,000G for 15 minutes in an MSE Micro Centaur microcentrifuge. The pellet was washed in 80% (v/v) acetone, and then resuspended in sample buffer for one- or two-dimensional electrophoresis.

(vi) Estimation of calmodulin in *in vitro* translation products

a) Affinity chromatography

Calmodulin was isolated from *in vitro* translation products by phenothiazine affinity chromatography as described in section H i c II. Material eluting from the column with EGTA was pipetted onto Whatman 3MM paper for estimation of (^{3}H) incorporation into TCA-precipitable material, and this was compared to the ^{3}H present in TCA-precipitable material loaded onto the column. A crude estimate of the relative abundance of calmodulin-like material in the *in vitro* translation products was obtained by estimation of the TCA-precipitable ^{3}H found in the EGTA eluate as a percentage of the total TCA-precipitable ^{3}H loaded on to the column.

b) Polyacrylamide gel electrophoresis

Calmodulin was tentatively identified on two-dimensional

NEPHGE-SDS polyacrylamide gels of *in vitro* translation products by a calcium-dependent electrophoretic mobility shift. Samples were solubilised for NEPHGE as described in section G iii a II, with either 10mM $CaCl_2$ or 15mM EGTA in the homogenisation buffer. SDS polyacrylamide gel electrophoresis was carried out in the second dimension in the presence or absence of 1.5mM EGTA in the electrophoresis buffer as described in section G iii b. A polypeptide migrating to pH 3.9 with an apparent molecular weight of 16,000 in the presence of calcium, but appearing at 21,000, 18,000 and 16,000 in the presence of EGTA was tentatively identified as calmodulin.

CHAPTER 3 : RESULTS

DEVELOPMENT OF EXPERIMENTAL PROCEDURES FOR INVESTIGATING THE BIOCHEMICAL COMPOSITION OF CELLS DURING DIFFERENTIATION IN THE PEA ROOT APEX

1. SEPARATION OF PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

A major part of this thesis is concerned with the analysis of protein composition during differentiation. As discussed in chapter 1, histochemical and enzymological techniques have several limitations as methods of demonstrating changes in protein content, and polyacrylamide gel electrophoresis was therefore employed as an initial means of examining proteins.

Polyacrylamide gel electrophoresis has several advantages as a preliminary method of analysing the protein composition of crude extracts. As total cellular protein may be examined, the range, extent and nature of protein changes during differentiation can potentially be indicated, and information can be obtained on the amount and rate of synthesis of proteins that occur even in extremely low concentration. Furthermore, it may indicate specific proteins that may be of interest, and can provide a means of isolating proteins or of further analysing the properties of individual proteins.

The development of suitable extraction procedures for polyacrylamide gel electrophoresis of total protein from pea root tissue is outlined in the following section.

A. TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Introduction

Two-dimensional polyacrylamide gel electrophoresis separates proteins on the basis of two independent parameters, molecular weight and charge, and has the capacity to resolve several thousand proteins on a single gel. It is potentially an extremely sensitive method for detecting changes in protein complement, and was therefore the main form of PAGE used to analyse proteins in the root apex.

The two-dimensional system of O'Farrell (1975) was used to separate proteins. This involves isoelectric focusing in the first dimension and separation by MWt in the second dimension. It was originally devised by O'Farrell for the separation of

bacterial proteins, and although it has been used successfully with a wide variety of plant and animal tissues, problems were encountered in the initial attempts to solubilise and separate proteins from the roots of pea seedlings. The development of electrophoretic procedures is therefore described below.

(ii) Separation of proteins in the first dimension by isoelectric focusing

a) Theory

Proteins for isoelectric focusing are solubilised and denatured in a high concentration of urea, a denaturing agent that destroys non-covalent interactions. This allows unfolding of the protein chain, exposing ionizable groups that contribute to the pI. Electrophoresis is then carried out in a gel which is relatively non-restrictive to protein migration containing a low concentration of acrylamide, and urea. A natural pH gradient is created electrophoretically in this gel by carrier ampholytes, and proteins ideally migrate to their pI within this gradient.

The position reached by a protein in IEF-PAGE is, however, dependent on a large variety of factors including the degree of denaturation and therefore exposure of dissociable groups that would contribute to net charge (Scanu *et al.*, 1973; Williamson *et al.*, 1973; Ui, 1971); amino acid modifications (Jacobs, 1973; Bobb, 1973; Hagel *et al.*, 1971; Gerding *et al.*, 1971; Williamson *et al.*, 1973; Robinson and Rudd, 1974); ion concentration (Robinson and Rudd, 1974); pH-stability and temperature stability of the polypeptide (Vesterberg, 1976); pH-mobility of the molecule (Rilbe, 1976); protein interactions (Grand, Naim and Perry, 1980); buffering capacity and therefore localized influence on the pH gradient of neighbouring proteins (Svensson, 1961; Rilbe, 1976); and the kinetics of pH gradient formation and decay (Chrambach and Baumann, 1976; Catsimpoolas, 1976). Any of these factors may produce artifacts, altering apparent pI or increasing or decreasing the apparent heterogeneity of a polypeptide during IEF.

Further problems arise due to the nature of isoelectric

focusing in polyacrylamide gels. As formation and decay of the pH gradient is a cooperative phenomenon with all amphoteric electrolytes including carrier ampholytes and proteins approaching their pI at characteristic individual rates and remaining in the steady state for different and limited durations, the development and decay of the gradient and focusing of proteins are dynamic, asynchronous, and are continually interacting. Formation and decay of the pH gradient and the focusing of individual proteins have complex kinetics, and isoelectric focusing remains largely empirical in practice (Catsimpoolas, 1976; Rilbe, 1976; Chrambach and Baumann, 1976; Baumann and Chrambach, 1975).

It may be seen therefore that the pI of a protein in isoelectric focusing refers to the pH region reached by the polypeptide after an arbitrary time under arbitrary conditions, and will be referred to as apparent pI or pI'.

b) Extraction procedures

In this project, two-dimensional PAGE was initially carried out as a means of separating polypeptides to indicate the degree and nature of changes in protein levels and protein synthesis during differentiation. To some extent, therefore, the absolute pI of any polypeptide was irrelevant, although useful in aiding identification. However, for comparison of different developmental stages, it was essential to know whether differences observed had been introduced during extraction or electrophoresis due either to differential modification or solubilisation of protein. It was therefore very important to minimise artifacts and to reproduce conditions as accurately as possible.

While conditions in O'Farrell IEF gels are devised to minimise production of artifacts, specific artifacts may arise in extraction. One major potential source of artifact production is the carbamylation of proteins by ammonium cyanate which is formed as a decomposition product of urea (Stark *et al.*, 1960; Ui, 1971; Edelstein *et al.*, 1972; Hagel *et al.*, 1971; Gerding *et al.*, 1971; Basha, 1979). Aqueous urea was therefore prepared below 20°C and stored at -20°C to minimise formation of cyanate; while samples were kept for a minimum time in

urea-containing buffers. Other artifacts particularly relevant to extraction of protein from pea roots are discussed below.

I. Solubilisation in urea, method 1.

Protein extraction from pea root was initially carried out by homogenisation of the tissue in the lysis buffer 'A' described by O'Farrell (1975), composed of 9.5M urea, 5% MESH, 2% (w/v) NP40 and 2% ampholines (1.6% pH5-7, 0.4% pH3-10). However, irrespective of the amount of protein loaded onto the gel, very few proteins could be visualised by Coomassie blue staining on either the first or second dimension gels, indicating that very little protein entered the first dimension gel. Proteins extracted by this procedure were generally low MWt and acidic. A gel of protein extracted from the apical 2 mm of pea root by this method is shown in Figure 3:1:1a.

Initial attempts to improve extraction centred around the possibility that homogenisation alone was insufficient to break down cell structure, and included repeated cycles of freezing and thawing in lysis buffer as described by O'Farrell (1975), sonication in lysis buffer, fractionation of cells,and acetone precipitation of proteins prior to solubilisation in lysis buffer. As these procedures were unsuccessful, it appeared that the lysis buffer was inadequate to fully solubilise proteins, or that some factor in the tissue was interfering in solubilisation.

Although urea was used as the denaturing agent, it is known that it only rarely produces completely randomised chains, and does not therefore act as a complete denaturing agent (Florini *et al.*, 1973; Williamson *et al.*, 1973; Bhakdi *et al.*, 1975). Membrane proteins in particular tend to be poorly solubilised by high concentrations of urea (Ames and Nikaido, 1976). It therefore seemed possible that aggregates of partially denatured proteins and organelles were interfering with entry of protein into the gel. In this context it was noted that Loening (1961) had observed that ribonucleoprotein particles composed largely of ribosome aggregates occur in homogenised sections of pea root, appearing as a white precipitate in the sample. As a

Figure 3:1:1. Two-dimensional polyacrylamide gel electrophoresis of pea root proteins: comparison of extraction procedures

Protein from sections of the apical 2 millimetres of pea root were extracted by different procedures as described in the text. 100µg of protein were then separated by isoelectric focusing or NEPHGE in the 1st dimension, and by molecular weight in the 2nd dimension.

a) Solubilisation in urea, method 1. Separation by isoelectric focusing in the 1st dimension.

b) Sonication in RNase. Separated by isoelectric focusing in the 1st dimension.

c) Solubilisation in urea, method 2. Separated by NEPHGE in the 1st dimension.

d) Sonication in 0.2% SDS. Separated by isoelectric focusing in the first dimension.

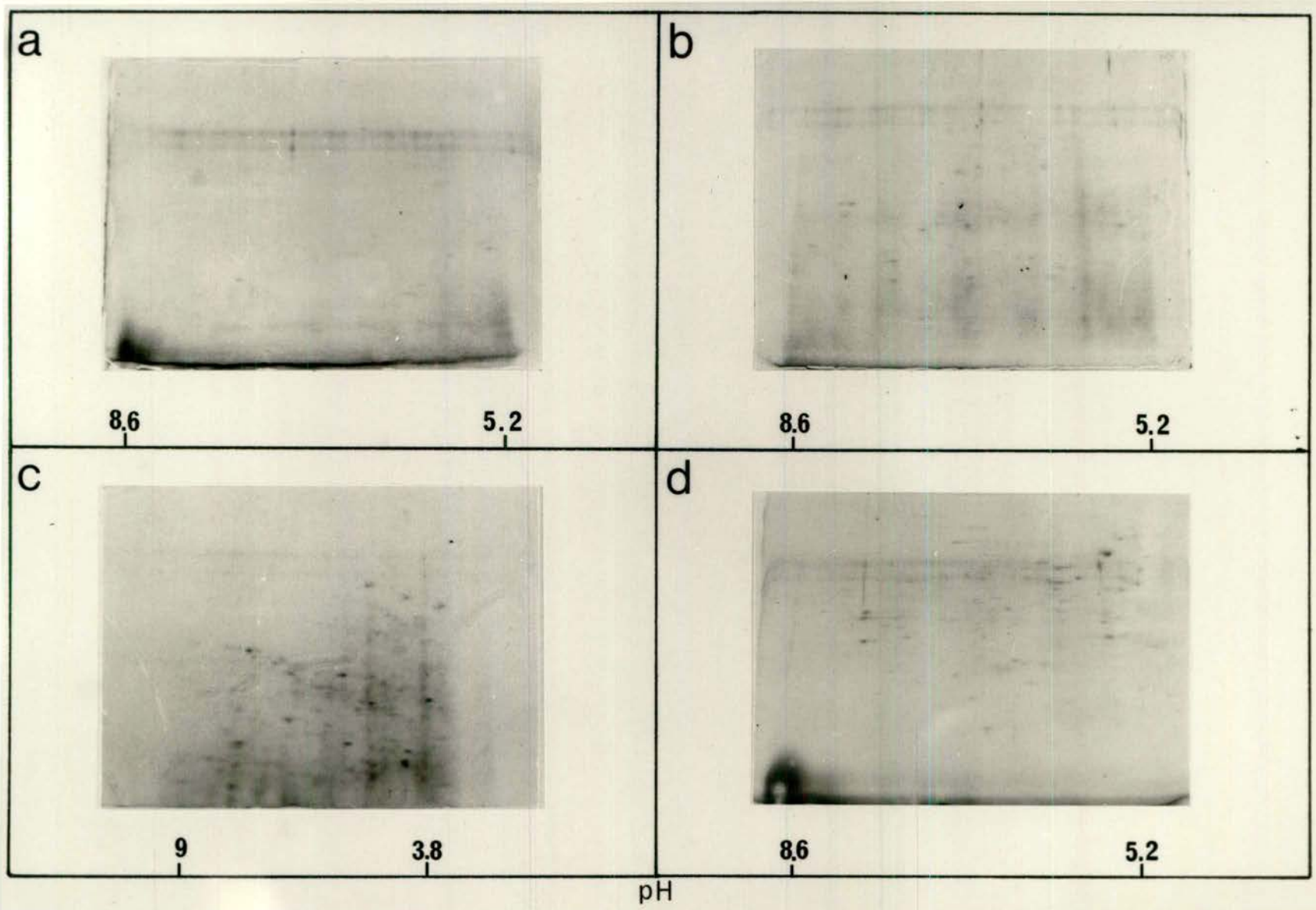


Fig. 3:I:I.

white precipitate was observed in the solution on top of the isoelectric focusing gels after electrophoresis, there was a possibility that aggregates of ribonucleoprotein particles interfered with entry of proteins to the gel. A further indication that nucleic acid-protein interactions may interfere in isoelectric focusing was obtained from the observation that basic proteins, which include ribosomal proteins and histones, were absent from the gel.

O'Farrell had found that nucleic acids in bacterial proteins interfered with isoelectric focusing by binding to basic ampholines which then, due to the highly ionic nature of the interaction, bound proteins. This resulted in severe streaking of the proteins across the gel when electrophoresed from the cathode to the anode. This form of interference was minimised in gels of pea root by electrophoresis routinely from the anode; however, the possibility remained that nucleic acids were interfering more directly with electrophoresis of pea root protein. To investigate the possibility of interference of nucleic acids and aggregation of ribonucleoprotein particles, the samples were treated with nucleases.

O'Farrell (1975) had devised a method for removing interference by nucleic acids, by sonication of the sample in RNase followed by treatment with DNase. Initially, therefore, pea root tissue was homogenised in the RNase-containing buffer 'B' of O'Farrell followed by addition of solid urea to 9M and one volume of lysis buffer. This resulted in entry of a considerably greater number of proteins into the gel indicating that there was indeed some interference by nucleic acids. Sonication of either a homogenate or whole tissue section in RNase-containing buffer yielded a far greater number of proteins on the gel, and a further improvement still was obtained by carrying out the full O'Farrell procedure with addition of DNase at 4°C immediately after sonication in RNase and before addition of urea. Optimum sonication time was found to be two three-second bursts at 4°C. Interestingly the white precipitate that formed on top of the gel during electrophoresis decreased according to the amount of protein entering the gel.

The composition of the precipitate was not investigated further, however, it appeared that it may have included

aggregates of ribonucleoprotein particles. Interference by nucleic acids, in particular RNA, and inadequate solubilisation by urea appeared both to be factors contributing to poor extraction of proteins from pea root by the original O'Farrell method.

Sonication with nuclease treatment was found to give reproducible results, and had the advantage that homogenisation was not required for extraction thus eliminating a significant source of protein loss during homogenisation of very small sections of tissue. This procedure was therefore used to investigate protein complement during differentiation in the pea root. The final procedure is described in chapter 2, section 2 G iii a, and a gel of protein extracted from the apical 2 mm of pea root by this procedure is shown in Figure 3:1:1b.

II. Solubilisation in urea, method 2

Entry of total protein and of individual species of proteins into the first dimension gel appeared to depend critically on extraction conditions. Optimum conditions for isoelectric focusing vary according to the individual protein, and in electrophoresis of a complex mixture of proteins the conditions will be sub-optimal for most proteins. As unfavourable conditions may result in precipitation, aggregation or modification of protein, further methods of extraction were sought both to increase the range of proteins observed, and to investigate further possible sources of artifacts.

A major possible source of interference in protein extraction was the pH of extraction and electrophoresis as many proteins may precipitate on exposure to a pH unfavourable to their individual requirements. Ampholytes were included in the sample buffer and overlay solution as well as the gel to extend the pH range above the gel and to protect the sample from extremes of pH; while urea prevented binding of acidic or basic proteins to oppositely-charged ampholytes (Kaplan and Foster, 1971; Williamson *et al.*, 1973). However, the pH gradient produced by the ampholine range used by O'Farrell was relatively narrow, extending in the gel from pH 5.2-8.6, and several problems might

have arisen from this. Apart from loss of stability or precipitation due to exposure to an unfavourable pH within the gel, it was also possible that acidic proteins excluded from the gel aggregated or precipitated at their pI on top of the gel thus inhibiting entry of other proteins to the gel. Nucleic acids remaining at the anode would also have a greater opportunity to bind proteins while in solution and could thus interfere with entry of proteins into the gel.

The pH range of ampholytes was therefore extended in the gel and in the sample and overlay solutions to 2% ampholines entirely of the range pH3-10, resulting in extension of the pH gradient to pH4.6-9.3. Homogenising pea root tissue under these conditions allowed entry of a considerably greater number of proteins into the gel than did homogenisation with the narrower pH range of ampholytes, thus demonstrating the importance of pH values in isoelectric focusing.

This method of protein extraction was found to give reproducible results and was therefore used in conjunction with NEPHGE or isoelectric focusing to observe proteins of a greater pI range than the previous method (described in section c)). An example of protein extracted from the apical 2 mm of pea root by this procedure is shown in Figure 3:1:1c.

III. Solubilisation in sodium dodecyl sulphate

In the previous methods, extraction and denaturation of protein was carried out with high concentrations of urea. However, as urea was not entirely satisfactory as a denaturing agent, a different method of protein denaturation was attempted.

Powerful denaturing conditions are achieved with heat treatment of a sample in dodecyl sulphate. This method is particularly useful with relatively insoluble proteins such as high MWt, hydrophobic, and intrinsic membrane proteins (Ames and Nikaido, 1976; Ames *et al.*, 1974; Rosenbusch, 1974; Helenius and Simons, 1975).

Sodium dodecyl sulphate is an anionic detergent. The SDS anions bind to proteins and swamp out intrinsic charge differences between proteins, subunits dissociate and disruption of hydrogen,

hydrophobic and disulphide linkages occurs. Proteins are negatively charged, and of a uniform charge surface density. Dodecyl sulphate may be removed by an anion exchanger in 6M urea (Wrigley, 1976) or by addition of the non-ionic detergent NP40 in urea (Ames and Nikaido, 1976).

Ames and Nikaido (1976) developed an extraction method using SDS for solubilisation of bacterial envelope proteins. This involved heating in 1% SDS with subsequent addition of urea to 9M and two volumes of O'Farrell lysis buffer containing NP40 increased to 8%. This method has the advantage of the higher solubilising power of SDS, while the removal of the dodecyl sulphate anion by NP40 in the presence of urea allows exposure of charged areas of the protein contributing to its pI. Theoretically, therefore, the proteins may then migrate according to their native charge in isoelectric focusing.

Extraction of protein from pea roots was therefore carried out according to the method of Ames and Nikaido (1976). This proved to be very successful at solubilising proteins, particularly for extraction of high MWt proteins that were absent when samples were treated with urea alone. However, protein streaking on the gels was severe, and attempts to decrease this effect by optimising removal of SDS from proteins by adjusting the ratio of SDS to NP40, or by decreasing the amount of SDS bound by reducing the concentration within the range allowing full SDS-protein binding (Pitt-Rivers and Impiombato 1968) to 0.2% SDS, were unsuccessful. However, a further modification involving sonication of the sample in SDS with subsequent heat treatment reduced streaking markedly while retaining all the protein observed in unsonicated samples. DNase treatment prior to addition of lysis buffer was a further improvement. The extraction procedure was then optimised, and the final extraction procedure involved sonication of the sample in 0.2% SDS with subsequent DNase treatment. This was followed by heating to 100°C for 30 seconds. The sample was then taken to 9M urea, and two volumes of lysis buffer added. An example of pea root protein extracted by this method is shown in Figure 3:1:1d.

Ames and Nikaido claimed that SDS was either completely removed from *Salmonella typhimurium* membrane proteins, or was removed to a fixed and reproducible extent by their method.

However, the electrophoretic pattern obtained from pea roots, although reproducible, was markedly different from that obtained by the previous two methods described. It is probable, therefore, that a substantial amount of SDS remained on proteins, modifying their apparent pI; or that irreversible change in pI has taken place as described by Wrigley (1976).

In spite of modifications of charge it was felt that sonication in SDS might be useful in observing proteins in pea root due to solubilisation of membrane and high MWt proteins, and as modification of pI might allow entry of proteins into the gel that had a native pI outside the pH range of the gel. This method was therefore also used in examining changes in polypeptides between different developmental stages. The method is described in chapter 2 section 2.G iii a.

c) Non-equilibrium pH gradient electrophoresis

Many of the proteins potentially of interest in differentiation are highly acidic or basic. Basic proteins include phloem proteins (pI' between 9.6 and 10.4 (Beyenbach *et al.*, 1974; Weber *et al.*, 1974)), histones of pI 10-11 (Savíc and Poccia, 1978), and ribosomal proteins. Acidic proteins include calmodulin (pI 3.9-4.3 (Klee, 1980)) and xylem proteins. It was therefore of interest to extend the range of proteins observed on the gels to include those of unusually high or low pI values.

Isoelectric focusing gels developed a gradient of pH range 5.2-8.6 when run from the anode using ampholines predominantly in pH range 5-7, and a gradient of pH 4.6-9.3 when using ampholines pH range 3-10. Electrophoresis of samples from the cathode resulted in a large pH shift to the acidic end to give a pH range varying from 2.4-6.5, thus being of little use in separating basic proteins. However, observation of proteins of a wide pI range was achieved by NEPHGE, a procedure devised by O'Farrell, Goodman and O'Farrell (1977) in which proteins are separated electrophoretically in a pH gradient as for isoelectric focusing, but electrophoresis is arrested before all proteins have migrated to their pI. If basic proteins are to be examined, electrophoresis may be carried out from the anode

so that basic proteins lead in the separation, and vice versa.

To investigate the optimum time of NEPHGE, samples were homogenised in lysis buffer containing pH3-10 ampholines as described in chapter 2, and electrophoresed for varying lengths of time from the anode. The pH gradient of the first dimension gels and final protein separation achieved on the second dimension gels were compared.

The outline of the pH gradient was found to be virtually completely formed within 500Vhours. Many proteins were observed at the acidic end of the gel at this stage. By 1000Vhours the gradient had become approximately linear and remained so until about 2000Vhours, after which the basic end began to collapse. During this period, many proteins remained in a similar position at the acidic end, presumably being acidic proteins that had migrated to their approximate pI' at an early stage; while many basic proteins migrated progressively towards the cathode. By 6,400Vhours, used for isoelectric focusing, decay of linearity of the pH gradient was occurring, the pH range had narrowed to pH 4.6-9.3, and loss of both highly acidic and basic proteins had occurred. The decay of the pH gradient has been commonly observed (Catsimpoolas, 1976; Baumann and Chrambach, 1975; Chrambach and Baumann, 1976) and is not entirely understood. Ampholine depletion of the gel is a major contributing factor (Baumann and Chrambach, 1975). The optimum time of electrophoresis for both separation of proteins and number of proteins observed on the gel was found to be 2000Vhours at 400V for 5 hours, resulting in a pH gradient of 3.8-9.

NEPHGE therefore increased the range of proteins observed on the second dimension gel by extending the pH range at the anode permitting the entry of more acidic proteins to the gel, and by arresting migration of basic proteins before they had reached their pI. It also improved the resolution of many basic proteins that were badly streaked on isoelectric focusing gels as a result of the collapse of the basic end of the pH gradient during isoelectric focusing. Separation of proteins was highly reproducible under identical electrophoretic conditions. NEPHGE of total protein and of the purified acidic protein, calmodulin, were carried out as described in chapter 2 section 2 G iii a. Pea root proteins separated by NEPHGE in

the first dimension are shown in Figure 3:1:1c.

d) Polymerisation of first dimension gels

It should be noted that although polymerisation with ammonium persulfate and TEMED or photopolymerisation with riboflavin and TEMED were both satisfactory methods for gels using predominantly pH5-7 ampholines, riboflavin was found to inhibit polymerisation in gels composed of pH3-10 ampholines. It was found necessary to use ammonium persulfate in conjunction with TEMED to polymerise these gels, and to use twice the concentration of that required for polymerisation of pH5-7 gels. This is possibly due to interference with polymerisation by basic ampholines (O'Farrell *et al.*, 1977).

Initially, riboflavin had been used in preference to ammonium persulfate as a polymerising agent, as persulfate, which can modify proteins by oxidation, is required to be removed by storing the gel overnight or by preelectrophoresis of the gel (Wrigley, 1976). Prerunning the gel is time consuming, and it was preferable to leave gels overnight if ammonium persulfate was used. However, as the gels had to be stored above 10°C to prevent crystallisation of urea, there was a risk of ammonium cyanate formation from urea decomposition. The patterns of proteins on gels that were prerun, not prerun and that were stored overnight were therefore compared. As no differences in the pattern were observed, it was concluded that cyanate and persulfate were not significant factors in causing artifacts. Gels polymerised with ammonium persulfate were therefore routinely made the day before they were required.

e) Extraction methods: modification of protein in different tissue types

The comparison of different developmental stages by two-dimensional PAGE relies on the absence of modification or on identical modifications and degree of solubilisation of proteins occurring in different tissues. This was examined by observing sections in different zones extracted and electrophoresed separately and together. No obvious differences could be

detected in protein patterns in zones homogenised and electrophoresed together from that expected by separate extraction and electrophoresis. Thus, if modification of pI does occur, there do not appear to be modifying influences in different tissues that produce different types of modification, although it is possible that differences in accessibility of modifying factors to proteins may occur in different sections. Furthermore, extractability of individual proteins may vary between sections. As this is much harder to examine, the amounts of individual proteins extracted by different extraction procedures have to be compared.

(iii) Separation of proteins in the second dimension by electrophoresis in SDS

Electrophoresis in the second dimension was carried out essentially according to O'Farrell (1975), a procedure based on that of Laemmli (1970). Exponential gradients of polyacrylamide concentration were found to produce a better distribution of protein than a single concentration gel. An exponential gradient of 10.5-15% polyacrylamide was therefore used for the second dimension of all two-dimensional gels. As described in section Bii, several proteins do not migrate according to MWt in SDS-PAGE. One protein chosen for further examination, calmodulin, was known to display a calcium-dependent mobility shift in PAGE, and this was confirmed under the conditions used here. The calcium concentration in the extraction buffer was therefore controlled during investigation of calmodulin by PAGE.

The final procedure for electrophoresis in the second dimension is described in chapter 2 section 2 G iii b.

B. ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Introduction

One-dimensional polyacrylamide gel electrophoresis theoretically separates proteins on the basis of one property. Separation may be carried out in non-denaturing gels, on the basis of MWt in the presence of SDS, or on the basis of charge by isoelectric focusing.

One-dimensional separation is of limited value in resolving

protein from complex mixtures, but is very useful for investigating properties of individual or isolated proteins, and for monitoring purity of samples during protein purification procedures. Non-denaturing PAGE is particularly useful for investigating protein binding characteristics.

One-dimensional polyacrylamide gel electrophoresis was therefore carried out where appropriate.

(ii) Separation of proteins by electrophoresis in the presence of SDS

a) Introduction

Polyacrylamide gel electrophoresis in the presence of SDS has several advantages that make it the most commonly used form of one-dimensional electrophoresis at present. SDS is a powerful solubilising agent being particularly useful for the extraction of hydrophobic proteins including intrinsic membrane proteins; it is less subject to production of artifacts than isoelectric focusing; samples may be stored for long periods without loss of stability, and it provides information on MWt.

SDS is an anionic detergent. Dodecyl sulfate anions bind to proteins and swamp out intrinsic charge differences between proteins, subunits dissociate and disruption of hydrogen, hydrophobic and disulphide linkages occurs. All proteins migrate as anions of uniform charge surface density, possessing an overall conformation close to a random coil. Migration rate in polyacrylamide gels then depends on the effective size of the complex.

However, although most proteins migrate according to their MWt, some do not. These include proteins with an unusual charge or conformation, or that contain carbohydrate constituents or unreduced sulfhydryl groups (Grefrath and Reynolds, 1974; Pitt-Rivers and Impiombato, 1968; Banker and Cotman, 1972). In some cases anomalous migration is probably due to an unusual conformation in SDS; or in the case of most glycoproteins, unreduced proteins (Pitt-Rivers and Impiombato, 1968) and some enzymes like glucose oxidase, papain and pepsin (Lambin, 1978), to an unusually low ratio of SDS binding to the protein on a weight basis (Pitt-Rivers and Impiombato, 1968); while migration of calmodulin is dependent on the presence of calcium in the sample.

Aggregation of proteins may arise from exposure of intrachain sulfhydryl groups by SDS or disruption of interchain disulphide linkages by MESH (Shapiro *et al.*, 1967) also giving misleading MWt values.

It has been observed that although many proteins do not migrate in a linear fashion with respect to MWt in a gel of a constant acrylamide concentration as the linear relationship is only valid within a limited range at any polyacrylamide concentration (Shapiro *et al.*, 1967), that gradient gels give a far more reliable indication of MWt, with a linear relationship between log (MWt) and log of polyacrylamide concentration reached by a protein (Lambin, 1978). This is described in chapter 2 section 2 G i.

b) Conditions of extraction and electrophoresis

Although certain proteins do not migrate according to MWt in SDS-PAGE, their migration rate will remain the same under identical electrophoretic conditions. The protein separation obtained in different gels will therefore be comparable provided electrophoresis conditions remain the same.

SDS-PAGE was carried out essentially as described by Laemmli (1970), and details are given in chapter 2. However, in view of the temperature dependence of SDS denaturation of many proteins (Helenius and Simons, 1975; Rosenbusch, 1974; Ames *et al.*, 1974), the effect of temperature on SDS protein extraction was investigated. A number of proteins were extracted in SDS at 20°C, however, many more proteins were extracted when the samples were heated to 100°C. As no difference in polypeptides appearing on gels was found to occur between 30 seconds and 10 minutes at 100°C, 2 minutes at 100°C was routinely used for extraction.

An exponential gradient of polyacrylamide concentration was found, in addition to providing a more reliable indication of MWt, to provide a much better distribution of pea root proteins than gels of a variety of single polyacrylamide concentration. An exponential gradient of 10.5-15% polyacrylamide was therefore used in all experiments. Conditions of one-dimensional SDS PAGE are described in chapter 2 section 2 G i.

(iii) Separation of proteins by non-denaturing polyacrylamide gel electrophoresis

In non-denaturing gels proteins are extracted and electrophoresed in conditions that will maintain their 'native' structure. To some extent migration is dependent on MWt as there appears to be a linear relationship for most proteins between migration distance of the protein in a gradient polyacrylamide gel and the square root of time of electrophoresis, unless electrophoresis is carried out near the pI of the protein (Lambin and Fine, 1979).

Electrophoresis in non-denaturing gels was initially carried out essentially as described by Amphlett *et al.*, (1976), and Perrie and Perry (1970). The gels consisted of 12.5% acrylamide, 80mM glycine, 25mM Tris pH8.6, and the stacking gel of 5% acrylamide, 80mM glycine, 25mM Tris pH 6.8. Electrophoresis buffer contained 25mM Tris pH 8.6, 80mM glycine. This procedure was found to be very unsatisfactory, possibly due to the presence of glycine in the stacking and separating gels which increased resistance during electrophoresis markedly, and which may interfere with its use as the slow migrating ion in the stacking of proteins.

It was found that following the procedure for SDS-PAGE but omitting the SDS gave satisfactory results. As for SDS gels, an exponential gradient of polyacrylamide was found to produce a better distribution than a single concentration gel. The final procedure is described in chapter 2, section 2 G ii.

C. SUMMARY

Methods for the extraction and electrophoresis of proteins from pea root have been developed for both two-dimensional and one-dimensional analysis.

Several problems were encountered in extraction of proteins for two-dimensional PAGE. A major problem was the interference by nucleic acids, and three methods were devised to overcome this problem. As each of these methods had further individual advantages and could be used to some extent to complement and check changes in protein content indicated by the other methods, all three methods were used to investigate protein changes during differentiation.

It was felt, however, that despite attempts to minimise production of artifacts and to check that modifying influences did not vary between sections, that the range of possible modifications and differences in extractibility of some proteins, and the lack of knowledge of identity and function of individual proteins on the gels limited the usefulness of two-dimensional PAGE without further investigation. It was felt that individual proteins appearing to vary between tissues by two-dimensional PAGE should be examined further. For reasons discussed in chapter 1 section 2 C ii, calmodulin and calmodulin-binding proteins were chosen to be observed in greater detail. The development of methods to isolate, identify and quantitate these proteins are described in sections 2 and 3 of this chapter.

3:2. DEVELOPMENT OF METHODS FOR THE ESTIMATION OF CALMODULIN IN THE ROOT APEX

Introduction

Several qualitative and quantitative changes in proteins were observed during differentiation in the pea root apex by separation on two-dimensional polyacrylamide gels. However, due to problems in interpreting apparent changes in protein complement as indicated by this method and in understanding the significance of such changes, it was thought that more detailed examination of individual proteins that appeared to vary would provide a more reliable and meaningful estimation of protein changes during differentiation. For reasons briefly described in chapter 1 section 2C the concentration, synthesis and function of calmodulin were investigated. This section describes the development of methods for determining the presence of calmodulin in pea root, and for estimating its concentration.

All the accepted methods for estimating calmodulin in a tissue have several difficulties connected with them, involving interference with the assay and extractability from the tissue. This becomes particularly problematical when the level of calmodulin in different tissues is to be compared. For this reason it is desirable that estimates of calmodulin should be obtained by more than one method. Calmodulin was therefore measured in the root apex by enzyme activation, polyacrylamide gel electrophoresis, and spectrophotometrically after purification to homogeneity, by absorbance at 276nm. In view of the problems apparently encountered by other workers in obtaining satisfactory antibodies to calmodulin, radioimmunoassay was not attempted.

Extraction of calmodulin is complicated by two main considerations. Firstly, it is found in both soluble and membrane fractions depending at least partially on the presence of calcium and calmodulin-binding proteins. Secondly, methods used to extract calmodulin may also extract calmodulin-like proteins. Several problems arose in the isolation, identification, and estimation of pea calmodulin. The attempts to

overcome these problems are described in the following 42 pages.

A. Purification of calmodulin from bovine brain

(i) Introduction

A satisfactory method for estimating the concentration of calmodulin in crude extracts is not available. Although radioimmunoassay and bioassay are both commonly used to measure calmodulin in such extracts both methods are subject to extensive interference, in particular by calmodulin-binding - and calmodulin-like - proteins. To some extent, problems involved in measuring calmodulin may be overcome by using purified calmodulin in the assays, and purified enzymes for activation studies.

In view of the problems involved in estimating calmodulin in cell extracts, it was therefore desirable to obtain purified calmodulin for bioassay and gel electrophoresis, and also for the investigation of conditions that may influence its behaviour during extraction and assay.

Calmodulin has been purified from or demonstrated to occur in several plant tissues although it has not been extracted from roots. I therefore felt that it would be useful to gain experience in purification and identification of calmodulin from a well characterised system before attempting to purify it from pea root tissue. Calmodulin is found in high concentration in neurosecretory cells where it may constitute up to 3% of cellular protein (Lin *et al.*, 1980; Watterson *et al.*, 1976), and high yields are readily available from such tissues. The protein from bovine brain in particular has been extensively characterised, and several methods have been developed for its purification to homogeneity. Calmodulin was therefore initially purified to homogeneity from bovine brain.

(ii) Extraction of calmodulin from bovine brain

Calmodulin was obtained from bovine brain acetone powder

by two methods, both involving phenothiazine affinity chromatography using fluphenazine coupled to Sepharose 4B by the bisoxirane method (Sundberg and Porath, 1974) according to Charbonneau and Cormier (1979). The first method, carried out by Dr. Trewavas, was that of Charbonneau and Cormier (1979) who obtained purified calmodulin from a variety of plant and animal tissues including pea seedling and porcine brain by their procedure. Purification involved affinity chromatography in the dark after simple homogenisation of the acetone powder in a calcium-containing buffer of 5mM $CaCl_2$, 50mM Hepes, 0.5M NaCl pH 8. After centrifugation of the homogenate for one hour at 100,000G, the supernatant was loaded onto a fluphenazine Sepharose 4B affinity column. Extensive washing of the column was carried out with this buffer to remove unbound proteins, until the absorbance at 280nm of the column effluent returned to the baseline value. Calmodulin was then eluted from the column with 10mM EGTA, and fractions that showed an increase in absorbance at 280nm were pooled. Calmodulin was then concentrated by ammonium sulphate precipitation (80% saturation), redissolved in sterile Tris buffer pH 8, and after dialysis against Tris buffer for six hours was stored at -20°C.

The second method was a slight modification of the method of Caldwell and Haug (1981a) as described in chapter 2 section 2 H i. This method improved upon the former by concentration of the sample for application to the affinity column, and by removal of a large number of contaminating proteins before affinity chromatography. The removal of these proteins was achieved by ammonium sulphate precipitation at neutral pH in the presence of EDTA. Calmodulin was then recovered from the ammonium sulphate supernatant by isoelectric precipitation at pH 4. After stirring for two hours the solution was centrifuged for 30 minutes at 12,000G, and the pellet resuspended in calcium-containing buffer. After dialysis to remove residual traces of ammonium sulphate, the solution was clarified by centrifugation at 100,000G for one hour, and applied to an affinity column in a calcium containing buffer. The column was then washed, and calmodulin eluted, concentrated and stored as before.

Both of these samples displayed properties identical to those

of calmodulin including molecular weight and isoelectric point, heat stability, calcium-dependent enzyme activation, phenothiazine inhibition, and behaviour on polyacrylamide gels. Neither sample contained any contaminating protein detectable by one- or two-dimensional polyacrylamide gel electrophoresis and the yield was therefore measured by absorbance at 276nm according to Anderson *et al.*, (1980).

Calmodulin obtained from brain by the method of Charbonneau and Cormier yielded 500µg/g acetone powder while 835µg/g acetone powder was obtained by the method of Caldwell and Haug. The specific activity of calmodulin obtained by the latter method for the activation of phosphodiesterase and NAD kinase was slightly higher than that of calmodulin obtained by the former. The reason for this is not clear, however, it may be due to an overestimation of calmodulin obtained by the method of Charbonneau and Cormier if aggregation of the protein had occurred. Aggregation of calmodulin is known to increase the absorbance at 276nm by an increase in light scattering, while decreasing the biological activity (Anderson *et al.*, 1980; Charbonneau and Cormier, 1979). Alternatively, as the procedure did not involve the removal of contaminating proteins prior to affinity chromatography it is possible that minor contamination of the sample with calmodulin-binding or calmodulin-like proteins may have occurred Co-elution of calmodulin-binding (Anderson and Cormier, 1978; Jamieson and Vanaman, 1979) and calmodulin-like proteins (Marshak *et al.*, 1981; Schleicher *et al.*, 1982a; Levin and Weiss, 1978) is known to occur in phenothiazine affinity chromatography of some samples. These proteins may be in too low a concentration to be detected by gel electrophoresis, but would interfere with the enzyme assays.

The lower yield of calmodulin is probably largely a result of extraction in calcium which will result in a relatively high proportion of calmodulin being retained in the particulate fraction associated to calmodulin-binding proteins bound to membranes. As conditions thereafter were not designed to prevent interference by calmodulin-binding proteins these proteins may also have interfered with the binding of calmodulin to the affinity column.

Bovine brain calmodulin was therefore finally purified in large amount by the method of Caldwell and Haug. The properties of this sample of calmodulin were investigated, in particular its behaviour under different conditions of extraction and electrophoresis in polyacrylamide gels and in enzyme activation, to identify conditions that could modify its behaviour. Modifications and precautions were made accordingly in the procedures for purifying and identifying pea root calmodulin. A description of the properties of calmodulin isolated from bovine brain is given below, and the properties summarised in table 3:2:2.

(iii) Identification of calmodulin from bovine brain

a) Introduction

Calmodulin-like proteins have been found in a variety of plant and animal species (Van Eldik *et al.*, 1980b; Chafouleas *et al.*, 1979; Wasserman and Smith, 1981; Schleicher *et al.*, 1982a). These proteins not only display one or more of the characteristics of calmodulin, but some of them may be purified by the methods used for the isolation of calmodulin. Therefore unless the identity of purified calmodulin is established by amino acid sequence, even if a protein has been purified to homogeneity it is necessary to determine its identity as calmodulin by a number of criteria.

Calmodulin has a number of unusual and distinctive properties which have been highly conserved throughout evolution, and these characteristics are very useful in its identification. The samples of bovine brain calmodulin were therefore examined in detail.

b) Molecular weight

Calmodulin is a low molecular weight monomeric protein. The amino acid sequence has been analysed from calmodulin obtained from a number of tissues, and the protein has been found to be comprised of 148 amino acid residues of a total molecular mass of 16,680 daltons (Watterson *et al.*, 1980). It appears to be monomeric as analysis by ultracentrifugation (Stevens *et al.*, 1976; Lin *et al.*, 1974) and equilibrium

sedimentation under non-denaturing conditions (Watterson *et al.*, 1976; Lin *et al.*, 1976) produce molecular weight estimates of 14,500-19,000 daltons. Analysis of molecular weight by gel filtration consistently produces anomalously high estimates of about 28,000-31,000 (Lin *et al.*, 1974; Muto and Miyachi, 1977; Dedman *et al.*, 1977) although calmodulin migrates according to its molecular weight under appropriate conditions in SDS polyacrylamide gels. However, calmodulin displays anomalous behaviour in polyacrylamide gels that appears, at present, to be unique among proteins. This provides a very useful means for its identification, and for distinguishing it from calmodulin-like proteins.

(I) The behaviour of calmodulin on SDS polyacrylamide gels

When the protein isolated from bovine brain was electrophoresed on SDS polyacrylamide gels with an exponential gradient of 10.5-15% acrylamide, it was found that the number of bands observed and their apparent molecular weight were entirely dependent on the amount of calcium or EGTA present. If the level of calcium was not controlled, a single band was seen to migrate with a mobility consistent with a molecular weight of 17,000 daltons. If EGTA was included in both the sample and electrophoresis buffers, however, a single more slowly migrating band was observed at 21-22,000 daltons. If EGTA was present in the sample buffer only, a band was present at 20-21,000 daltons, and occasionally a minor band also at 22,000. On the addition of calcium to the sample buffer, however, a major band migrated rapidly to the anode to a position consistent with a molecular weight of 15-16,000 daltons, two minor more slowly migrating bands also being observed at 17,000 and 18,500 daltons. If calmodulin was electrophoresed with calcium and EGTA in neighbouring wells, the bands in the tracks containing calcium were distorted. In this case, bands migrated to approximately 17,000 and 18,500 daltons in the centre of the track but joined to a single point at the edge of the track at 22,000 daltons, directly adjacent to calmodulin in the track containing EGTA. The results are shown in figure 3:2:1.

Figure 3:2:1. Calcium-dependent mobility shift of bovine brain calmodulin in one-dimensional polyacrylamide gels

One-dimensional SDS polyacrylamide gel electrophoresis of bovine brain calmodulin purified by the procedure of Caldwell and Haug (1981a) was carried out as described in chapter 2 section 2 G i.
Lanes A-C and G-J are 10.5-15% exponential polyacrylamide gradient gels. Lanes D-F are 12.5% polyacrylamide gels.

A. 15mM EGTA added to sample buffer.

B. Molecular weight markers.

C. 10mM $CaCl_2$ added to sample buffer.

D. 15mM EGTA added to the sample buffer. Sample electrophoresed adjacent to a well containing $CaCl_2$.

E. 10mM $CaCl_2$ added to the sample buffer. Sample electrophoresed adjacent to a well containing EGTA.

F. Molecular weight markers.

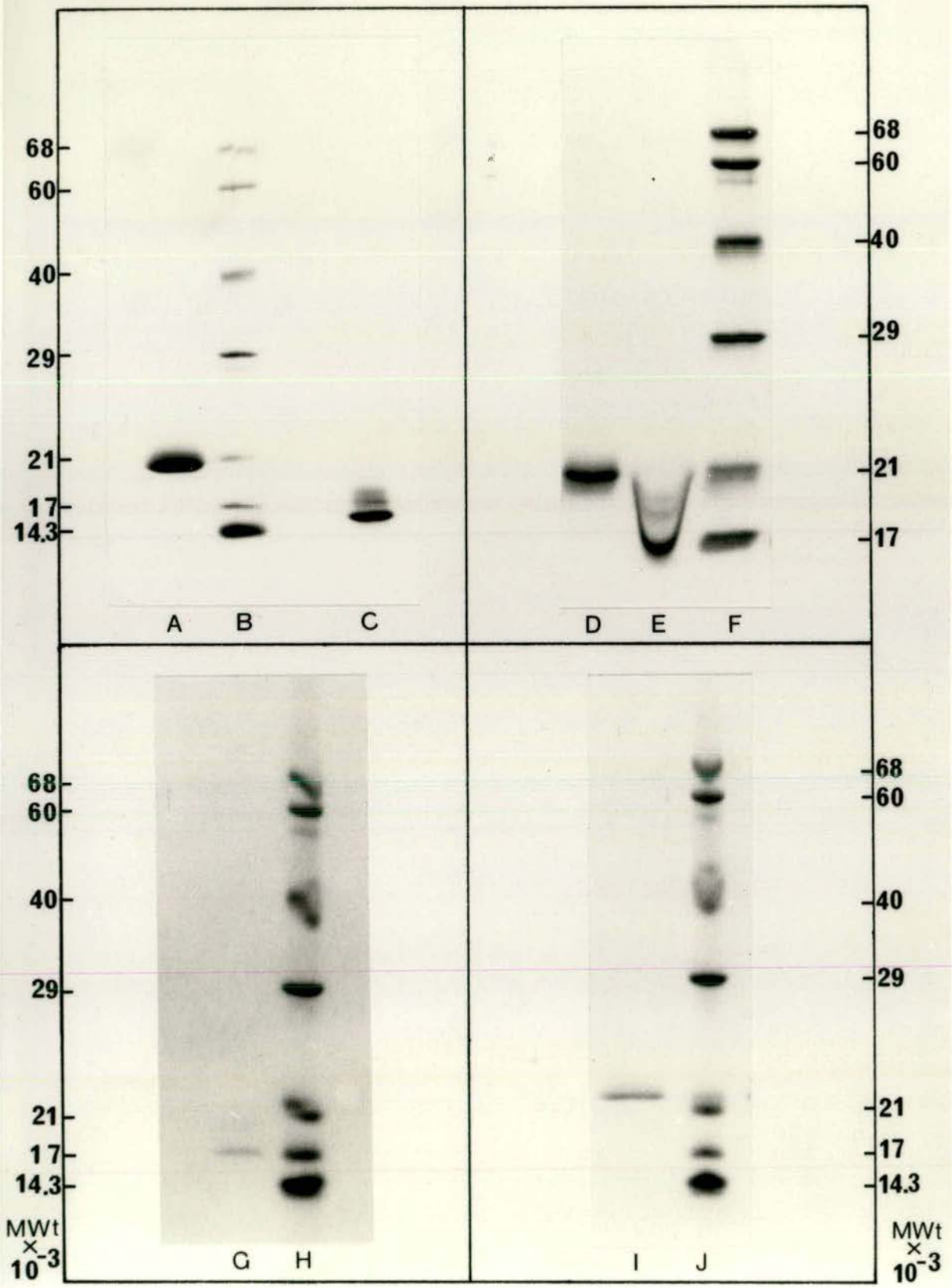
G. Neither $CaCl_2$ nor EGTA were added during electrophoresis.

H. Molecular weight markers.

I 15mM EGTA added to sample buffer. 1.5mM EGTA added to the electrophoresis buffer.

J. Molecular weight markers.

Fig.3:2:I.



This calcium-dependent electrophoretic mobility shift is entirely consistent with the behaviour of purified calmodulin, and the phenomenon has been well documented. In general it has been found that calmodulin appears to migrate with a molecular weight of 15,000-18,000 daltons if the calcium concentration is not controlled during extraction and electrophoresis (Jarrett and Penniston, 1978; Stevens *et al.*, 1976; Watterson *et al.*, 1976; Van Eldik *et al.*, 1980a; Lin *et al.*, 1974). If calcium is included during electrophoresis the mobility is increased and calmodulin migrates with an apparent molecular weight of 13,500-15,000 daltons, while in the presence of EGTA or EDTA it migrates with a slower mobility of 18,000-21,000 for bovine brain calmodulin (Van Eldik *et al.*, 1980a; Burgess *et al.*, 1980).

Although less frequently reported there have been several instances where calmodulin has appeared as more than one band simultaneously during polyacrylamide gel electrophoresis. Esnouf *et al.*, (1980) routinely observed five bands on polyacrylamide gels of calmodulin from various sources. There are also several reports of a more slowly migrating band appearing in SDS gels on heating (Burgess *et al.*, 1980) or after storage of calmodulin (Burgess *et al.*, 1980; Jarrett and Penniston, 1978; Van Eldik and Watterson, 1979) although no loss in biological activity occurs. It has also been found by Burgess *et al.*, (1980) that the number of bands is dependent on the level of calcium present, as described above for bovine brain calmodulin isolated by the method of Caldwell and Haug (1981a), with one band occurring in the presence of EGTA, and three more rapidly migrating bands in the presence of calcium.

This phenomenon was originally suggested by Klee *et al.* (1979) to be a result of EGTA binding to calmodulin as relatively large amounts of chelator were found to be required to produce the mobility shift. However, Burgess *et al.* (1980) subsequently found that at 0.1mM $CaCl_2$, four $^{45}Ca^{2+}$ ions bound per molecule of calmodulin in the presence of 0.1% SDS, and therefore suggested that the higher molecular weight band seen in the presence of calcium chelators represented calcium-free calmodulin, and that the lower molecular weight bands reflected different amounts of calcium bound to calmodulin. The capacity for binding calcium

under denaturing conditions may be altered by heating or by storage of calmodulin, and would thus explain the multiple bands observed under different electrophoretic conditions, without loss of biological activity.

The results obtained by Burgess *et al.* for calcium-dependent migration of calmodulin are similar to those that I obtained for bovine brain calmodulin isolated by the method of Caldwell and Haug, except that one, two or three bands could be discerned on some gels depending on the presence of calcium. However, it should be noted that calcium was not included in the electrophoresis buffer or the gels as it caused precipitation of lauryl sulphate, nor was EGTA included in the gels as it inhibited polymerisation. This will reduce the concentration of calcium and EGTA during electrophoresis, presumably therefore permitting partial binding of calcium to calmodulin as well as both the fully bound and calcium free states. As calmodulin may bind up to four calcium ions with the change in conformational state occurring in two stages according to the number of calcium ions bound (Klee *et al.*, 1977; Seamon, 1980), binding to calcium in the presence of SDS might also occur in two stages. Resistance to denaturation would probably differ accordingly, resulting in anomalous migration and multiple bands in SDS polyacrylamide gels.

A calcium-dependent shift in electrophoretic mobility has been observed in a few other proteins, all calcium-binding proteins. Troponin-C (Van Eldik *et al.*, 1980b; Klee *et al.*, 1979a), a calmodulin-like protein from *Chlamydomonas* flagella (Van Eldik *et al.*, 1980b) and calcineurin B (Klee *et al.*, 1979a), are the only other known proteins to display this property. However, the shift in these proteins is of the order of 500-1,000 daltons as opposed to over 4,000 daltons for calmodulin, and may not always be detectable, even in gels of high acrylamide concentration (Burgess *et al.*, 1980).

Although the protein isolated from bovine brain appeared to be calmodulin purified to apparent homogeneity, there was a possibility that it was contaminated with a calmodulin-like protein which co-migrated with calmodulin in the presence of EGTA, but not in the presence of calcium. Troponin-C, for example, a calcium-binding protein found in muscle cells that

has a striking degree of structural and functional homology with calmodulin, co-migrates with calmodulin in 10% SDS polyacrylamide when calcium levels are not controlled (Stevens *et al.*, 1976). However, the calcium-dependent mobility shift of troponin-C is far lower than that of calmodulin (Van Eldik *et al.*, 1980b). The proteins can also usually be resolved on polyacrylamide gels of concentrations of 12.5% or higher on either SDS gels, where calmodulin runs with a mobility of about 500-1,000 daltons lower apparent molecular weight than troponin-C (Dedman *et al.*, 1977; Van Eldik *et al.*, 1980b) or on non-denaturing gels (Van Eldik *et al.*, 1980b; Stevens *et al.*, 1976), where troponin-C migrates faster in the presence of calcium than EGTA whereas calmodulin migrates more slowly.

Although the isolation procedure employed distinctive characteristics of calmodulin, several calmodulin-like proteins have acidic isoelectric points, and may be isolated by phenothiazine or naphthalenesulfonamide affinity chromatography in a calcium-dependent manner, such as troponin-C (Levin and Weiss, 1978; Gariepy and Hodges, 1983), a calmodulin-like protein from *Chlamydomonas* (Van Eldik *et al.*, 1980b; Schleicher *et al.*, 1982), the calcium-binding brain protein S100 (Marshak *et al.*, 1981; Endo *et al.*, 1981), and possibly troponin-C like proteins from non-muscle cells (Kretsinger, 1980). As the purification procedure for bovine brain calmodulin involved isoelectric precipitation and phenothiazine affinity chromatography, further tests on the identity and purity of calmodulin had to be carried out as co-purification of a calmodulin-like protein such as S100 or troponin-C like proteins may have occurred.

(II) <u>The behaviour of calmodulin on non-denaturing gels and two-dimensional polyacrylamide gels</u>

Calmodulin from bovine brain was therefore electrophoresed on two-dimensional gels, with an exponential gradient of 10.5-15% polyacrylamide in the SDS dimension to separate low molecular weight proteins. No additional polypeptides were observed in the isoelectric focusing dimension although calcium-dependent

Figure 3:2:2. Calcium-dependent mobility shift of bovine brain calmodulin in two-dimensional polyacrylamide gels

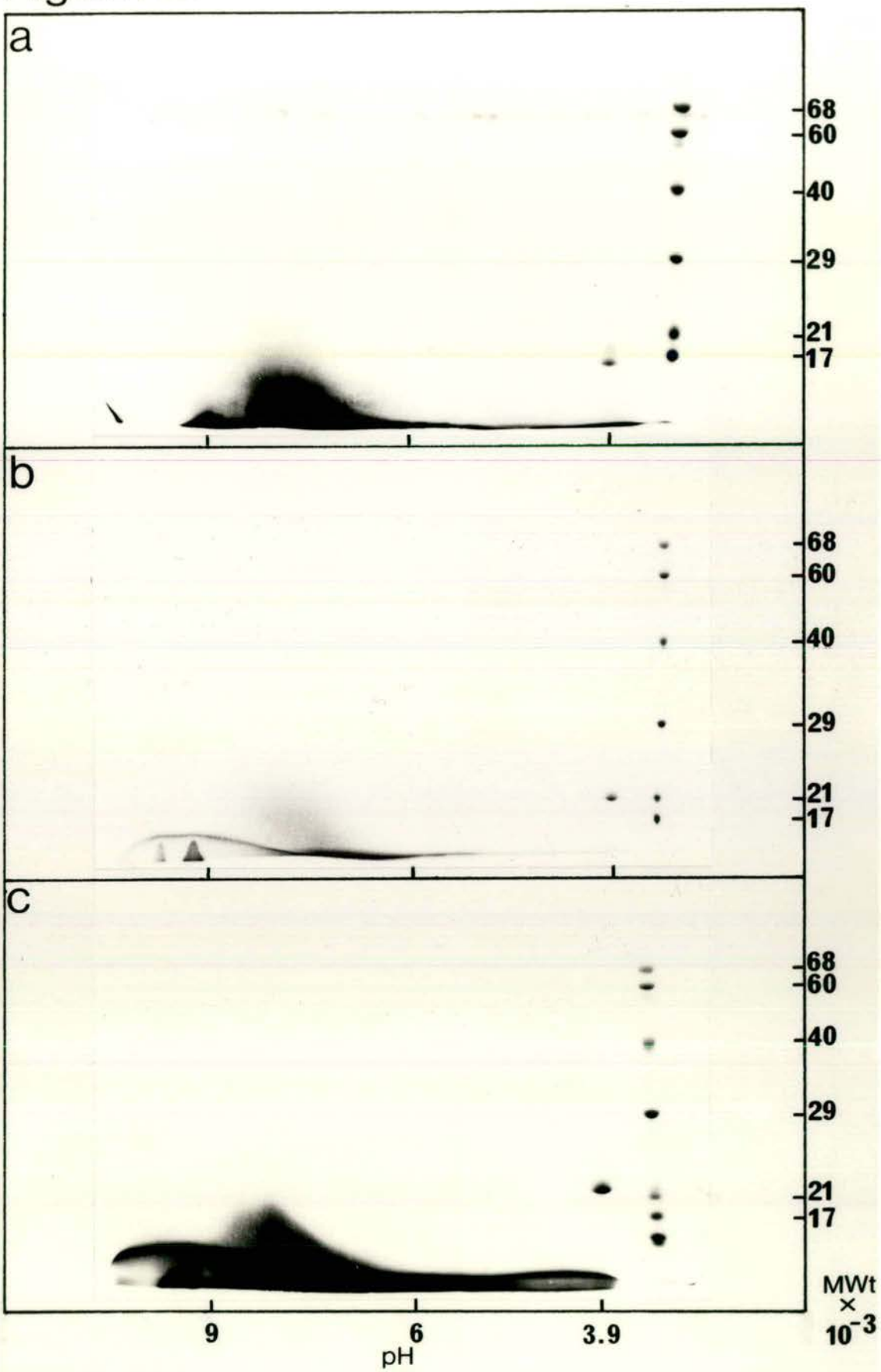
Two-dimensional polyacrylamide gel electrophoresis of bovine brain calmodulin purified by the procedure of Caldwell and Haug (1981a) was carried out by NEPHGE in the first dimension and SDS electrophoresis in the second dimension as described in chapter 2 section 2 G iii.

a) 10mM $CaCl_2$ was added to the sample buffer.

b) 15mM EGTA was added to the sample buffer

c) 15mM EGTA was added to the sample buffer.
1.5mM EGTA was added to the SDS electrophoresis buffer in the second dimension.

Fig. 3:2:2.



alteration of mobility still occurred in the SDS dimension (figure 3:2:2). As other proteins known to bind to phenothiazine columns in the presence of calcium have slightly different isoelectric points from calmodulin, it is very unlikely that the multiple bands are contaminating protein.

Electrophoresis was also carried out in non-denaturing gels as calmodulin is the only known protein to display a calcium-dependent mobility shift in the reverse direction of that in SDS gels. Troponin-C is the only other protein known to display a mobility shift in non-denaturing gels, but it migrates with a faster mobility in the presence of calcium, whereas calmodulin migrates with a slower mobility (Grand *et al.*, 1979; Burgess *et al.*, 1978).

When the protein isolated from bovine brain was electrophoresed on exponential gradient non-denaturing polyacrylamide gels, one band was observed if calcium was included in the sample buffer. If calcium was replaced with EGTA, the polypeptide migrated more rapidly to the anode, with several minor more slowly migrating bands following. Again distortion of the bands occurred if calmodulin containing calcium and EGTA were electrophoresed in neighbouring wells. Results are shown in fig.3:2:3. Watterson *et al.* (1980) similarly found several bands in non-denaturing gels that did not appear on SDS or 6-8M urea gels. They suggested therefore that this was due to aggregation of calmodulin. Certainly, as the major band of bovine brain calmodulin in the presumably calcium-free state, in EGTA, migrated more rapidly than fully calcium-bound calmodulin, while additional minor bands observed with EGTA migrated more slowly than fully calcium-bound calmodulin it is possible that the multiple bands are not due to different degrees of calcium binding but rather to aggregation. If this is the case, it indicates that calcium may protect calmodulin against aggregation, possibly connected with neutralisation of negative charges and with an increase in helical structure which occurs on binding calcium (Klee, 1977). It seems probable that the difference in migration of calmodulin in calcium and EGTA buffers is due to a different conformational state and surface charge induced by calcium, but that multiple bands may represent, at least partly, the aggregation of calmodulin.

Figure 3:2:3. Calcium-dependent mobility shift of bovine brain calmodulin in non-denaturing polyacrylamide gels

Non-denaturing polyacrylamide gel electrophoresis of bovine brain calmodulin purified by the procedure of Caldwell and Haug (1981a) was carried out as described in chapter 2 section 2 G iii.

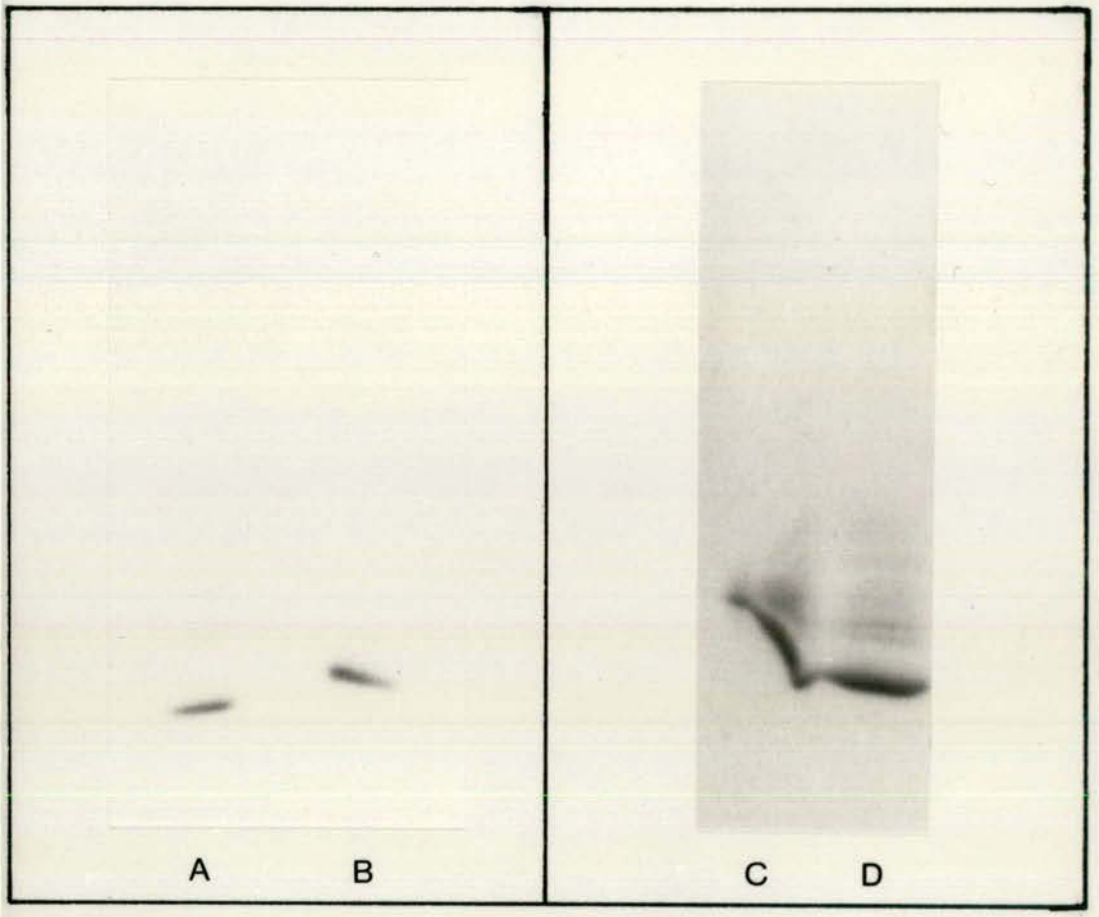
A. 15mM EGTA was added to the sample buffer.

B. 10mM $CaCl_2$ was added to the sample buffer.

C. 10mM $CaCl_2$ was added to the sample buffer. The sample was electrophoresed immediately adjacent to a well containing EGTA.

D. 15mM EGTA was added to the sample buffer. The sample was electrophoresed immediately adjacent to a well containing $CaCl_2$.

Fig. 3:2:3.



(III) Summary

The unique characteristics of the calcium-dependent mobility shift in SDS and non-denaturing gels may be used as a reliable, if subjective, method of identifying calmodulin on polyacrylamide gels.

From characteristics of migration on SDS, non-denaturing and two-dimensional polyacrylamide gels, it appeared that the protein isolated from bovine brain by the method of Caldwell and Haug (1981a) was homogeneous calmodulin. As both the apparent molecular weight and number of bands observed during polyacrylamide gel electrophoresis depended on the degree of calcium bound to calmodulin, the behaviour of calmodulin on these gels emphasises the need for caution in interpreting results obtained from electrophoresis.

c) Isoelectric point

Calmodulin has a preponderance of negatively charged amino acids, and the high ratio of combined aspartic and glutamic acids to lysine and arginine (Klee, 1977; Dedman *et al.*, 1977) account for its low isoelectric point. Estimates obtained for its pI' during isoelectric focusing vary between 3.9 when measured in a sucrose-pH gradient (Dedman *et al.*, 1977) and 4.3 in a urea-pH gradient (Lin *et al.*, 1974). Other estimates lie between this range (Stevens *et al.*, 1976).

The slight differences in pI' estimates are probably due to a variety of factors. These will include differences in the degree of denaturation of calmodulin in sucrose and urea and therefore of exposure of dissociable groups contributing to pI'; differences in estimation of the pH gradient, which will depend on the presence of urea (Bull *et al.*, 1964); the method of measurement of the pH gradient particularly in urea gels; the presence of calcium as calcium neutralises negative charges (Seamon, 1980) and stabilises calmodulin to denaturation; and differences in electrophoretic times as electrophoresis is not always carried out for long enough to produce isoelectric focusing conditions.

It was noted that there are several factors that will influence the apparent isoelectric point of calmodulin when measured in urea gels. Calmodulin is resistant to denaturation

even in 6-8M urea (Brostrom and Wolff, 1981) while calcium further stabilises calmodulin to urea denaturation (Walsh *et al.*, 1979). The mobility of calmodulin in urea gels would therefore be expected to be dependent on the concentration of urea and the presence of calcium or EGTA, and indeed calmodulin has been found to have a greater mobility in the presence of calcium than its absence in 6M urea gels (Walsh *et al.*, 1979; Amphlett *et al.*, 1976). As calmodulin is not completely denatured at 6M urea in the presence of calcium, it is probable that this reflects differential chain unfolding. However, as it is essentially unfolded in 8-9M urea whether calcium is present or not (Walsh *et al.*, 1979) it was felt that the use of high urea concentrations in isoelectric focusing of bovine brain calmodulin might produce complete denaturation of the molecule irrespective of the presence of calcium.

Although normally present as a single band, calmodulin has been reported to be present as two bands in isoelectric focusing. Van Eldik and Watterson (1979a) observed that different preparation and storage conditions resulted in a change in apparent isoelectric point, as well as in mobility in SDS as described in the previous section. They observed that two bands present in sucrose-pH isoelectric focusing gels, the major band at pI 4-4.2 and a minor band at pH 3.7-3.9, altered in intensity during storage of calmodulin, the latter becoming stronger while the former faded. They noted also that although one band was present in SDS gels when fresh samples were electrophoresed, that a second band appeared when older samples were run, but they could not tell if the two bands seen on SDS gels corresponded to those on isoelectric focusing gels. It should be noted, however, that Van Eldik and Watterson did not control the level of calcium in the isoelectric focusing gels, nor did they measure the pI' under denaturing conditions. The presence of two bands on the isoelectric focusing gels is therefore likely to reflect calmodulin in different degrees of denaturation, or different amounts of calcium bound to calmodulin, thus producing different pI' values as a result of a change in conformation and of exposure of dissociable groups.

As migration of calmodulin in isoelectric focusing gels

therefore appeared to be dependent both on the levels of calcium and on the strength of denaturing conditions, precautions were taken to avoid incomplete denaturation in the measurement of bovine brain calmodulin pI' by the use of strong denaturing conditions. The isoelectric point of calmodulin was therefore estimated by isoelectric focusing in 9.5M urea as described in chapter 2. As shown in figure 3:2:2, under these conditions, bovine brain calmodulin appeared to migrate as a single polypeptide with a pI' of 3.9 irrespective of the presence of calcium or EGTA.

d) Enzyme activation

Calmodulin-dependent enzyme activation is neither tissue- nor species-specific. The calmodulin-dependent isozymes of bovine heart phosphodiesterase and pea NAD kinase were therefore used to assay the activating ability of the protein isolated from bovine brain. Activating ability was compared with that of bovine heart calmodulin obtained from Sigma.

(I) Phosphodiesterase activation

The calmodulin-dependent form of phosphodiesterase is an enzyme that will hydrolyse both cAMP and cGMP. It has a low affinity for cAMP and a higher affinity for cGMP, however, the V_{max} for cAMP hydrolysis is higher than that for cGMP. Phosphodiesterase binds calmodulin in the presence of calcium, and this complex (Teshima and Kakiuchi, 1974; Lin *et al.*, 1975; Klee *et al.*, 1979b) at saturation reduces the k_m for cAMP and raises the V_{max} for cAMP and cGMP hydrolysis (Teo *et al.*, 1973; Lin *et al.*, 1974; Van Eldik and Watterson, 1979b). As the increase in rate of hydrolysis is also more pronounced with cAMP, calmodulin changes the relative rate of hydrolysis in favour of cAMP.

Calmodulin-dependent 3':5'- cAMP phosphodiesterase obtained from Sigma was assayed by the method of Cheung (1971) as described in chapter 2 section 2 I i. Under the conditions

described, 0.005 units[1] of phosphodiesterase solution hydrolysed 0.9nmoles cAMP/minute in the absence of calcium and calmodulin. The addition of the purified protein from bovine brain resulted in a 6-7 fold increase in activity over basal to 5.7nmoles cAMP/minute when fully activated with 50-100ng of protein. Half maximal activation over basal activity was achieved at 20 ng, while half maximal activation of the fully active phosphodiesterase was reached at 16ng. By contrast, over 300ng of bovine heart calmodulin were required to half maximally activate fully activated phosphodiesterase. Activation of phosphodiesterase was dependent on the presence of calcium. The results are shown in figure 3:2:4.

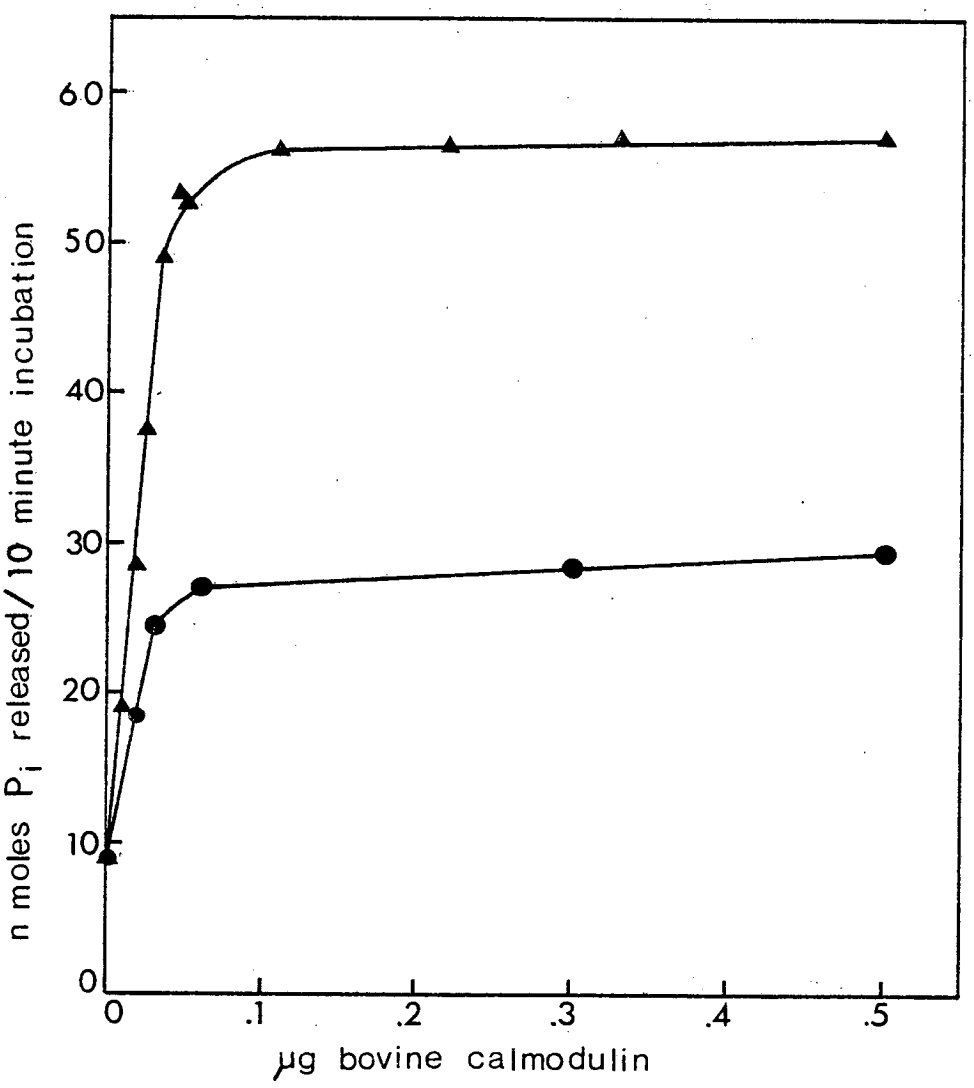
The activation of phosphodiesterase by the isolated brain protein was entirely consistent with the behaviour of calmodulin although its specific activity was sixteen times higher at 50,000 units/mg protein than that of bovine heart calmodulin obtained from Sigma at 3,125 units/mg protein. This seems unlikely to be due to co-purification of activators of phosphodiesterase other than calmodulin by the method of Caldwell and Haug as they would be unlikely to be co-purified (Liang *et al.*, 1981; Klee *et al.*, 1979b; Pichard and Cheung, 1977; Cheung, 1967; Dedman *et al.*, 1979b; Kretsinger, 1980) or would have lower specific activities than calmodulin (Dedman *et al.*, 1979b; MacManus, 1981). Furthermore, neither one- nor two-dimensional polyacrylamide gel electrophoresis detected any contaminating protein. The difference also seems unlikely to be due to a difference in activating abilities between bovine heart and brain calmodulins, as this has not been reported elsewhere. However, Sigma obtained their calmodulin by the method of Teo *et al.*, (1973). This procedure does not employ affinity chromatography, and could result in the co-elution of acidic low molecular weight proteins with calmodulin. Although Teo *et al.* (1973) found that the protein was homogeneous by one-dimensional polyacrylamide gel electrophoresis, they used an acrylamide concentration of 7.5% which is inadequate to resolve

[1] 1 unit of phosphodiesterase is defined as the amount that will hydrolyse 1μmole of 3':5'-cAMP to 5'AMP/minute under the conditions specified. 1 unit of calmodulin is the quantity required to produce half maximal activation of 0.005 units of phosphodiesterase.

Figure 3:2:4. Activation of calmodulin-dependent bovine heart 3':5'-cAMP phosphodiesterase by bovine calmodulin

Phosphodiesterase activity was assayed by the method of Cheung (1971) as described in chapter 2 section 2 I i. Activation of phosphodiesterase was achieved using either bovine brain calmodulin (——▲——) obtained by the method of Caldwell and Haug (1981a) as described in chapter 2 section 2 H i, or bovine heart calmodulin (——●——) obtained from Sigma. The results are expressed in terms of inorganic phosphorus released from 5' AMP by 5' nucleotidase activity. 5' AMP is produced during the assay from cAMP hydrolysed by phosphodiesterase.

Fig. 3:2:4.


60
50
40
30
20
10
0
n moles P_i released/10 minute incubation
0
.1
.2
.3
.4
.5
μg bovine calmodulin

proteins of similar molecular weight to calmodulin such as troponin-C. Nor did they observe the sample on two-dimensional gels which would separate two polypeptides of similar molecular weight but different isoelectric point. Although a heat treatment stage was included thus removing many contaminating proteins, a heat stable inhibitor of calmodulin is known to exist (Sharma *et al.*, 1978b). As this is a high molecular weight protein it is also unlikely to have been co-purified, however, it is possible that other heat stable inhibitors exist. Alternatively, the sample of bovine heart calmodulin may have undergone aggregation during storage, thus reducing its biological activity.

(II) NAD kinase activation

Calmodulin-dependent NAD kinase was partially purified from pea shoots and separated from calmodulin by the method of Anderson *et al.* (1980), and assayed by a modification of the method of Muto and Miyachi (1977) as described in chapter 2. Calmodulin-dependent activation of this enzyme occurs in a similar manner to that of phosphodiesterase, requiring the prior binding of calcium to calmodulin.

Under the conditions specified, no enzyme activity could be detected in the absence of calmodulin and calcium. Full activation of 0.005 units[1] of partially purified NAD kinase was reached at 4 µg bovine brain calmodulin isolated by the method of Caldwell and Haug with the phosphorylation of 5 nmoles NAD to NADP/minute thirty hours after harvesting the tissue for enzyme extraction. Half maximal activation occurred at 600ng calmodulin. Activation was dependent on the presence of calcium. The results are shown in figure 2:6.

Bovine brain calmodulin isolated by the method of Charbonneau and Cormier had a lower specific activity, 1.6 µg being required for half maximal, and 10µg for full activation. As mentioned in

[1] 1 unit of calmodulin is defined as the amount required to produce half maximal activation of 0.005 units of NAD kinase. 1 unit of NAD kinase is the amount that will convert 1 µmole of NAD to NADP/ minute when fully activated. The specific activity of the NAD kinase 30 hours after harvesting was 0.05 units/mg protein.

section 2 A ii, this difference may be due to aggregation of calmodulin, to minor contamination by calmodulin-binding proteins or to co-purification of calmodulin-like proteins in the latter method. Co-purification of calmodulin-binding proteins could either interfere indirectly with the assay by binding to calmodulin in the presence of calcium, or directly by inhibiting calmodulin (Sharma *et al.*, 1978b, 1979; Klee *et al.*, 1979a; Wang and Desai, 1977; Wood *et al.*, 1980; Wallace *et al.*, 1980).

(III) Phenothiazine inhibition of enzyme activation

Phenothiazines are a class of antipsychotic drug that have been found to bind specifically to calmodulin in a calcium-dependent manner, resulting in the inhibition of its biological activity. The specificity of binding has been used extensively, not only for the affinity isolation of calmodulin, but also to investigate its biological function, and to demonstrate indirectly its presence in a tissue.

Calmodulin has been found to have both specific, high affinity, calcium-dependent binding sites, and non-specific, low affinity, calcium-independent binding sites. There are one to three calcium-dependent drug binding sites on calmodulin according to the phenothiazine involved (Levin and Weiss, 1979). The nature of binding to calmodulin is poorly understood, but is thought to involve both hydrophobic interaction between the phenothiazine nucleus and a non-polar region of calmodulin (Prozialeck and Weiss, 1982a, 1982b; Gopalakrishna and Anderson, 1982), and electrostatic interaction between a positively-charged amino group on the drug and a negatively-charged residue on calmodulin (Prozialeck and Weiss, 1982a). All proteins so far examined appear to have low affinity calcium-independent binding sites for phenothiazines (Levin and Weiss, 1977). Only calmodulin appears to have the high affinity calcium-dependent binding sites, with the exception of troponin-C (Gariépy and Hodges, 1983; Levin and Weiss, 1978).

Phenothiazines at low concentration bind specifically to

calmodulin in the presence of calcium, while in its absence binding is of the same order as that to other proteins (Levin and Weiss, 1977). Binding to other proteins in the presence of calcium does not reach the same level as to calmodulin until about 100 μM trifluoperazine (Levin and Weiss, 1977) at which stage binding to calmodulin is independent of calcium. Troponin-C is the only known exception, binding to the phenothiazine trifluoperazine as strongly as calmodulin at concentrations of the drug over 100 μM in the presence of calcium (Levin and Weiss, 1978), although binding is lower below 10 μM trifluoperazine.

Calmodulin-dependent enzyme activation may therefore be specifically inhibited by low concentrations of trifluoperazine in the presence of calcium. The effect of trifluoperazine on the ability of calmodulin from bovine brain to activate the enzymes phosphodiesterase and NAD kinase was investigated.

Trifluoperazine did not inhibit unactivated phosphodiesterase until concentrations over 100 μM were reached. However, activation of both phosphodiesterase and NAD kinase was greatly inhibited at low concentrations of the drug that had virtually no effect on the unactivated enzyme. In the presence of saturating levels of 10 units (200ng) of bovine brain calmodulin, the I_{50} for the activation of 0.005 units of phosphodiesterase was 12 μM trifluoperazine. Activation was completely inhibited by 50 μM trifluoperazine. Activation of 0.005 units of NAD kinase by a saturating level of 10 units (6μg) of calmodulin was inhibited almost totally by 80μM trifluoperazine, with an I_{50} of 22μM. The results are shown on figure 3:2:5.

The concentration of trifluoperazine required to inhibit the enzyme activation by the bovine brain protein and the form of the dose-response curve is consistent with that expected of calmodulin (Weiss *et al.*, 1974; Jarrett *et al.*, 1982; Levin and Weiss, 1976).

(IV) Thermal stability

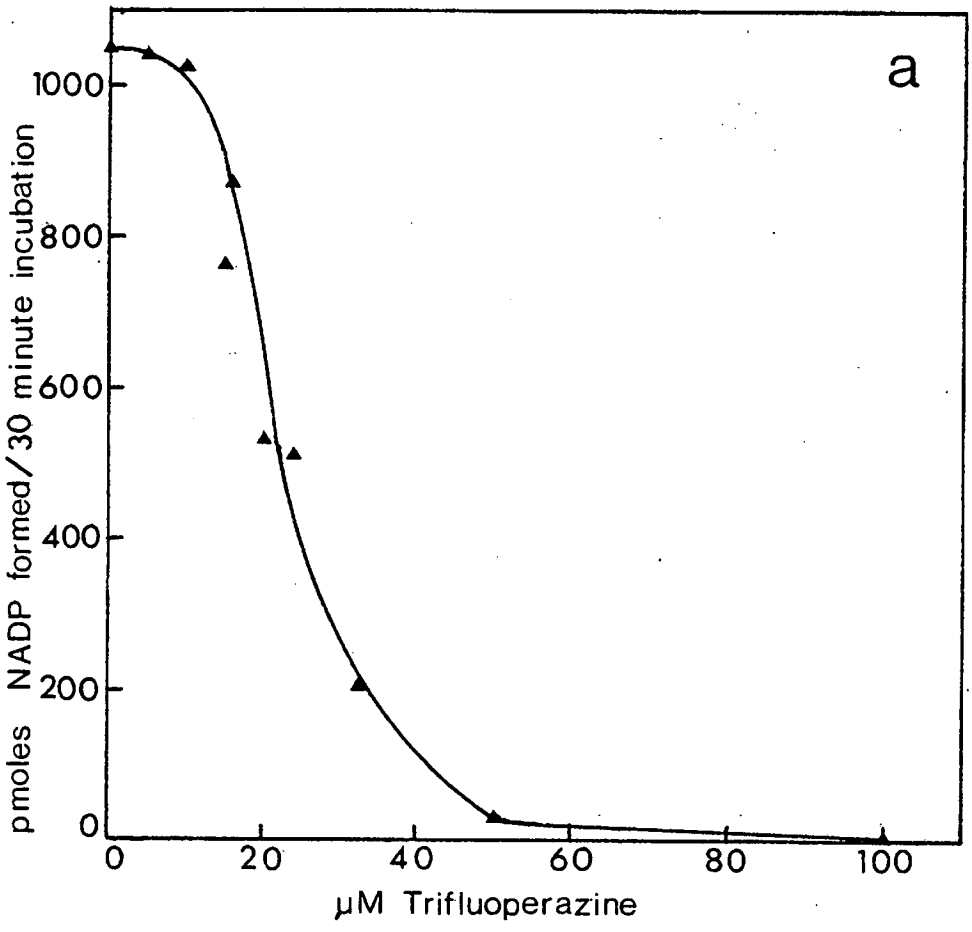
The enzyme activating ability of the bovine brain protein was not reduced by heating to 100°C for 3 minutes. This is consistent with the behaviour of calmodulin which can normally

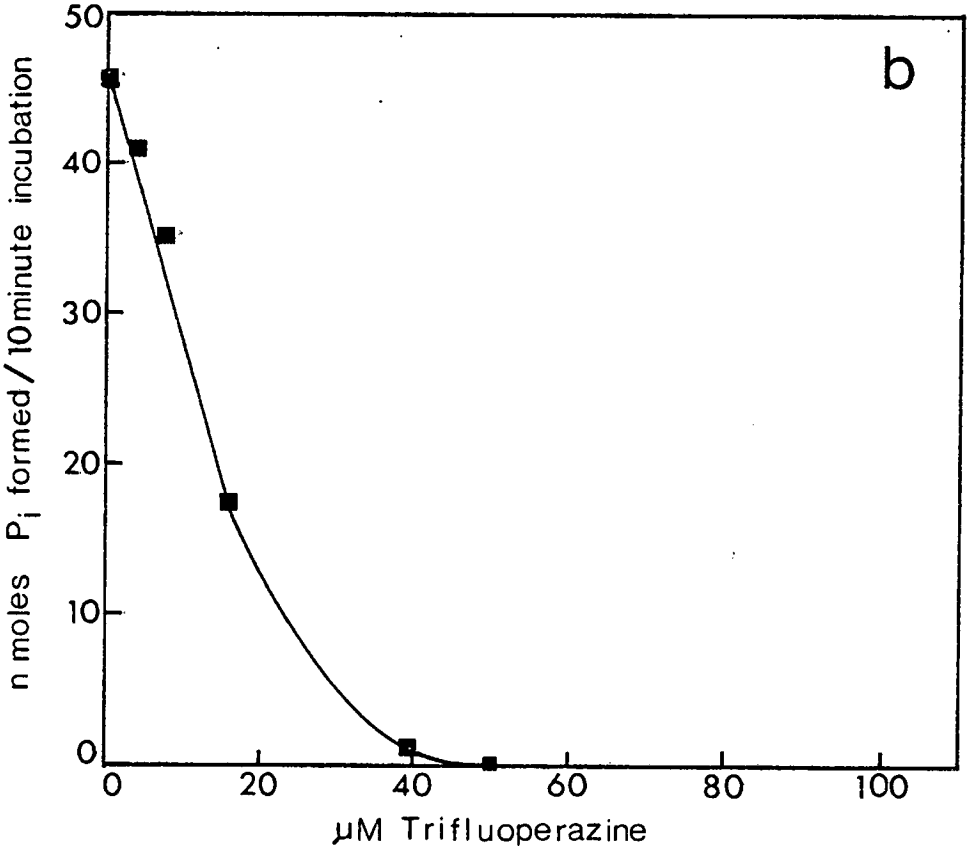
Figure 3:2:5. Phenothiazine inhibition of phosphodiesterase and NAD kinase activation by bovine brain calmodulin

a) Calmodulin-dependent NAD kinase was partially purified by the procedure of Anderson *et al.* (1980) as described in chapter 2 section 2 H ii a. The sample was completely inactive in the absence of calmodulin, and was fully saturated with 10 units of bovine brain calmodulin purified by the method of Caldwell and Haug (1981a). Inhibition of enzyme activation was carried out by dark incubation of fully activated NAD kinase with trifluoperazine.

b) Bovine heart calmodulin obtained from Sigma was fully activated with 10 units of bovine brain calmodulin purified by the method of Caldwell and Haug (1981a). Phenothiazine inhibition of enzyme activation was carried out using trifluoperazine incubated under dark conditions with fully saturated phosphodiesterase. The results show inhibition of the calmodulin-dependent activity of phosphodiesterase.

Fig. 3:2:5.


a
1000
800
600
400
200
0
pmoles NADP formed/30 minute incubation
0
20
40
60
80
100
μM Trifluoperazine


b
50
40
30
20
10
0
n moles P_i formed/10minute incubation
0
20
40
60
80
100
μM Trifluoperazine

withstand temperatures of 100°C for 5 minutes with only minimal loss of biological activity (Dedman *et al.*, 1977; Lin *et al.*, 1974).

(iv) Summary

Calmodulin was isolated from bovine brain by two methods. The method of Caldwell and Haug (1981a) was found to produce a higher yield of calmodulin of higher specific activity than that obtained by the method of Charbonneau and Cormier (1979). Both samples appeared to be homogeneous as analysed by one- and two-dimensional polyacrylamide gel electrophoresis, and their behaviour was consistent with that expected of calmodulin by a variety of criteria. The properties identical to those of calmodulin are a) molecular weight in SDS polyacrylamide gels, b) isoelectric point of 3.9, c) calcium-dependent electrophoretic mobility shift in both SDS and non-denaturing gels, d) heat stability, e) activation of the calmodulin-dependent cAMP phosphodiesterase, f) activation of the calmodulin-dependent isozyme of plant NAD kinase, g) calcium-dependent enzyme activation, h) inhibition of enzyme activation at low concentrations of phenothiazines, i) calcium-dependent binding to a phenothiazine affinity column. There does not appear to be any contamination of the calmodulin obtained by the method of Caldwell and Haug either by calmodulin-binding or calmodulin-like proteins as discussed in the preceding sections, although there may be some minor contamination of the sample obtained by the method of Charbonneau and Cormier.

Bovine brain calmodulin was therefore isolated by the method of Caldwell and Haug. This calmodulin was used to calibrate the activation of phosphodiesterase and NAD kinase for use in the assay for calmodulin in pea root. It was also used to investigate the behaviour of calmodulin during extraction from pea root tissue and its subsequent behaviour on polyacrylamide gels and in enzyme activation.

B. Purification of calmodulin from pea root

(i) Introduction

As indicated in the previous section, estimation of calmodulin by biological activity is subject to a variety of interfering factors, in particular calmodulin-binding - and calmodulin-like - proteins. Purification of calmodulin and of the enzymes used for its assay may overcome these problems to some extent. However, as many of these proteins may be isolated by similar extraction conditions to those for calmodulin, care has to be taken in extraction as interference may be aggravated by their co-purification.

The concentration of calmodulin in plant tissues has been found to be far lower in general than in animal tissues. Charbonneau and Cormier (1979), for example, purified 6.8 mg calmodulin/kg dry peanut seed, and estimated the original concentration to be 42 mg/kg. By their procedure, therefore, under the assumption that protein constituted about 28% of the weight of dry peanut seed, extractable calmodulin was approximately 0.002% of protein content, while total calmodulin was approximately 0.015% of protein content. This represents 0.1% of soluble protein after heat treatment. Similar results were apparently obtained for pea seedlings. If the concentration of calmodulin in pea roots was of the same order as that in peanut seed on a percent of total protein basis, then approximately 1.4-14µg total calmodulin/g fresh weight could be expected depending on the zone in the root, while 0.2-2µg/g fresh weight could be expected after purification.

Calmodulin was to be examined in small root sections of 1mm or less, each weighing approximately 1mg. It was therefore desirable that the assay systems were capable of detecting nanogram quantities of calmodulin, and the extraction method designed to maximise yield while minimising contamination. The NAD kinase assay system of Muto and Miyachi (1977) which could initially detect only millimolar quantities of NADP was therefore modified as described in section 3:3 to become 10^6 times more sensitive to NADP. The final conditions in the NAD kinase assay permitted detection of 1-2 picomoles of NADP and could

reasonably detect as little as 50ng of bovine calmodulin. The phosphodiesterase assay could detect less than 5 ng of bovine calmodulin. Two-dimensional polyacrylamide gel electrophoresis could readily detect 300 ng of bovine brain calmodulin when stained with Coomassie blue.

(ii) Extraction of calmodulin from pea tissue

a) Calmodulin in crude homogenates. Method 1

To obtain a rough estimate of the amount of calmodulin present in pea roots, ten sections of the apical 10mm weighing 75mg were homogenised in four volumes of the buffer used by Charbonneau and Cormier for calmodulin extraction, 25mM Tris, 2% PVPP, pH 8, and calmodulin was assayed by the activation of phosphodiesterase and NAD kinase in the presence of 0.1mM calcium. The samples were either used directly after centrifugation for 30 minutes at 10,000G, or were heated to 100°C for 2 minutes before centrifugation to remove heat-labile and calmodulin-binding proteins. However, no activation of either enzyme could be detected when amounts of the homogenate equivalent to 7-75mg pea root were assayed. As Muto and Miyachi (1977) had obtained readily detectable amounts of NAD kinase activator in crude homogenates using similar extraction conditions from less than 30mg of pea seedling, and had found pea seedling a relatively good source of activator compared to a variety of other plant species, it was not clear whether the lack of activation was due to absence or to a low concentration of calmodulin in root tissue, to poor solubilisation, to interference by or modification by some component of the homogenate, or to a low activating ability of the calmodulin in pea root. I therefore attempted to investigate some of these possibilities.

b) Extraction of calmodulin by the method of Charbonneau and Cormier. Method 2

Charbonneau and Cormier (1979) had successfully extracted calmodulin from pea shoot tissue, and I had already used their method to obtain relatively high yields from bovine brain purified to apparent homogeneity. Initially, therefore, I attempted to isolate calmodulin from pea shoot tissue by their

procedure to compare the sensitivity of the assay system for bovine calmodulin with that for plant calmodulin, and to examine whether it might be a suitable method for extracting pea root calmodulin. The first stage of this method involved partial purification of pea seedlings by the method of Anderson and Cormier (1978), with subsequent purification by affinity chromatography using fluphenazine-Sepharose 4B.

For this procedure, 300g of etiolated pea shoot tissue were homogenised in three volumes of 25mM Tris, 2% PVPP, pH 8. After straining through two layers of cheesecloth followed by centrifugation at 12,000G to remove PVPP and cell debris, the supernatant was taken to 50% saturation with ammonium sulphate. After equilibration the pellet was dissolved in and dialysed to an electroconductivity equivalent to 0.1M NaCl against a buffer containing 0.1mM EGTA, 20mM Tris, 0.1M NaCl pH 8. The dialysate was applied to a DEAE cellulose column and protein was eluted with a gradient of 0-0.6M NaCl. The electroconductivity of the eluted fractions was measured to determine NaCl concentration, and the fractions tested for presence of calmodulin after heat treatment by activation of phosphodiesterase and NAD kinase.

Some calmodulin-independent NAD kinase activity was found in the salt gradient from 0.1-0.3M NaCl (figure 3:3:1). However, although calmodulin had been found by Anderson and Cormier to peak at 330mM NaCl, no activation of the calmodulin-dependent enzymes was achieved with any of the heated fractions throughout the entire salt gradient (figure 3:3:1). As it was possible that some component interfering with enzyme activation had been co-purified or that calmodulin had remained bound to a calmodulin-regulated protein, the entire gradient was applied, after addition of calcium to 0.1mM, to a phenothiazine affinity column pre-equilibrated with 0.5mM $CaCl_2$, 10mM Tris pH 7 according to the method of Charbonneau and Cormier. Extensive washing in calcium-containing buffer with 0.5M NaCl to remove contaminating proteins was carried out until the A_{280} had returned to the baseline value. The material bound to the column in a calcium-dependent manner was eluted with 5mM EGTA and was concentrated by ammonium sulphate precipitation (80% saturation). The pellet was dialyzed against 20mM Tris pH 8 and stored at -20°C.

No protein could be detected by one-dimensional polyacrylamide gel electrophoresis and no activation of phosphodiesterase or NAD kinase could be detected. The procedure was repeated with light grown tissue, but again calmodulin could not be detected. Application of the void volume to the affinity column in addition to the material eluted with the salt gradient did not result in detection of calmodulin.

c) Modification of the extraction procedure of Charbonneau and Cormier to remove calmodulin-binding proteins. Method 3.

It was possible that calmodulin-binding proteins precipitated with calmodulin by ammonium sulphate were co-purified during ion exchange chromatography and interfered with its detection by enzyme assay at this stage. These proteins might then interfere with the binding of calmodulin to the phenothiazine affinity column. Although it is known that some proteins will bind to a phenothiazine column along with calmodulin, presumably being capable of remaining attached to calmodulin bound to phenothiazine, it has recently been discovered that phenothiazines will not dissociate preformed phosphodiesterase-calmodulin-calcium complexes (Saitoh *et al.*, 1982) indicating that preformed calmodulin-calcium-calmodulin-binding protein complexes may prevent binding of calmodulin to phenothiazine affinity columns. I therefore attempted to minimise this source of interference by removing a large number of contaminating proteins, including heat labile calmodulin-binding proteins, by heat treatment. Only one calmodulin-binding protein is known to be heat stable (Sharma *et al.*, 1978b, 1979) and this is not known to be found in plant tissues. It is therefore unlikely that any calmodulin-binding proteins would remain active after heat treatment. Although Anderson and Cormier have an optional heat treatment stage after ion exchange chromatography, I decided to heat the sample after ammonium sulphate precipitation in an attempt to increase solubilisation of calmodulin attached to membranes as well as to reduce interference by calmodulin-binding proteins. The method of Charbonneau and Cormier was therefore modified by heating the sample for 2 minutes at 100°C after dialysis of the ammonium sulphate precipitate against EGTA to avoid trapping calmodulin during denaturation of calmodulin-binding proteins.

After removal of precipitated proteins by centrifugation, the 15,000G supernatant was loaded directly on to the affinity column without passage through the ion exchange column.

From A_{276} values this procedure extracted 8µg calmodulin/g fresh weight of pea, representing about 0.12% of total protein. It therefore seems probable that calmodulin-binding proteins, or some heat-labile component in the sample may have interfered with affinity chromatography.

However, there was a discrepancy in the concentration of calmodulin as estimated by A_{276} values and that estimated by NAD kinase activation. Moreover, the specific activity of enzyme activation of this sample appeared to be much lower than that of bovine calmodulin. Ten times the amount of pea shoot calmodulin from A_{276} values was required for half maximal activation of phosphodiesterase, and 2.5 times the amount for NAD kinase activation. Certainly part of the difference in specific activity may be due to differences in activating ability of pea and bovine calmodulins, but the high estimate of calmodulin by A_{276} data indicates that at least part of the discrepancy between the estimates of calmodulin by the two methods may be due to overestimation of pea calmodulin by A_{276} values, to modification of activating ability during extraction, or to an artifact of the system. As it was possible that the partially purified NAD kinase contained some components that affected the activating ability of plant and animal calmodulins to a different extent, I attempted to purify NAD kinase to homogeneity by calmodulin-affinity chromatography as described in chapter 3, section 3. However, the yield was too low and, as the enzyme was so unstable, the procedure was too long to be suitable for use in enzyme activation studies. When analysed by polyacrylamide gel electrophoresis the sample was found to contain several proteins that had co-eluted with NAD kinase. As these proteins presumably bound to calmodulin in a calcium-dependent manner they may have included a protein differentially affecting plant and animal calmodulins. As a result of the length of the procedure and of the presence of additional calmodulin-binding proteins in the sample, this more highly purified sample of NAD kinase was not used for comparison

of activating abilities of plant and animal calmodulins.

When calmodulin obtained by this procedure was electrophoresed on polyacrylamide gels, no protein was observed despite high loadings as estimated by A_{276} values. There was therefore a possibility that protease activity was occurring in the sample which would lower the apparent specific activity of enzyme activation. Addition of protease inhibitors to the extraction and purification buffers did not, however, alter the specific activity of enzyme activation.

The low specific activity of pea calmodulin is also unlikely to be due to aggregation of calmodulin as, despite loading of up to 75µg of protein from A_{276} values, no protein was visible on one- or two-dimensional gels.

It is possible, however, that calmodulins from bovine and pea tissues have different activating abilities. Although Anderson *et al.* (1980) and Charbonneau *et al.* (1980) found that apparently equal amounts of plant and porcine brain calmodulin were required for half maximal activation of identical amounts of phosphodiesterase, recent results (Cormier *et al.*, 1982; Jarrett *et al.*, 1983) indicate that plant calmodulin is 7-fold more effective in activating plant NAD kinase, while certain plant calmodulins are one to two orders of magnitude less effective in activating phosphodiesterase and myosin light chain kinase than mammalian calmodulins (Grand *et al.*, 1980). As my own results indicate that there is indeed a difference between bovine and pea calmodulins in activating ability for phosphodiesterase and NAD kinase, it is possible that the anomalous results are at least partly due to a far lower specific activity of bovine calmodulin for the activation of NAD kinase, and a higher specific activity for the activation of phosphodiesterase. The amount of protein in pea calmodulin estimated by A_{276} values may be an overestimate due to contamination of the sample with material absorbing at this wavelength. Alternately, calmodulin may have broken down into low molecular weight fragments as described by Charbonneau and Cormier (1980) reducing its activating ability 30-fold, and additionally resulting in rapid electrophoresis in polyacrylamide gels and loss to the anode buffer.

It appeared from these results that pea shoot calmodulin

may have been modified during extraction, or that it may have a different activating ability from that of bovine calmodulin. However, even from the lower estimation of calmodulin obtained from NAD kinase activation data, rather than A_{276} values, the amount extracted from pea shoot appeared to be comparable to that obtained by Charbonneau and Cormier for peanut seed on a protein basis, as shown in table 3:2:1.

The procedure was therefore repeated using 300 sections of root tissue from different zones, with a smaller 1 ml affinity column. However, enzyme activating ability could not be detected in any of the fractions eluted from the affinity column with EGTA, although the A_{276} values again indicated that a substantial amount of material had been eluted from the column. However, a small amount of activating ability was found to be present in the heated fraction prior to application to the affinity column, the equivalent of 7 µg of bovine brain calmodulin/g pea root. This represents 0.01% of total protein. As Charbonneau and Cormier found that only 25% of the calmodulin loaded onto an affinity column could be detected in the EGTA eluate, the estimate of calmodulin concentration was again similar to the values obtained by Charbonneau and Cormier on a cellular protein basis.

d) Modifications of the extraction procedure to increase solubilisation and recovery of calmodulin. Methods 4 and 5.

Extraction methods were further investigated to examine the possibilities of poor solubilisation of calmodulin, and incomplete precipitation with ammonium sulphate.

There are conflicting reports of the ammonium sulphate concentration and pH value required to precipitate calmodulin. The ammonium sulphate fractions used to obtain calmodulin include a 50% pellet at neutral pH (Anderson and Cormier, 1978; Anderson *et al.*, 1980), a pH 4 pellet from a 55-60% ammonium sulphate pH 7 supernatant (Van Belle, 1981; Endo *et al.*, 1981; Watterson *et al.*, 1976; Burgess *et al.*, 1980; Caldwell and Haug, 1981a; Yamanaka and Kelly, 1981), a 90% pellet at pH 6 (Dedman *et al.*, 1977a), a pH 4 50% ammonium sulphate pellet (Charbonneau and Cormier, 1979) and a pH 4 supernatant (Beale *et al.*, 1977). Although I had been using the fraction of Anderson and Cormier

for extraction from pea seedlings, it seemed possible that the conditions of precipitation were not appropriate for the cultivar Feltham 1st.

The initial extraction of calmodulin during homogenisation may also have been poor. It has been found by many workers that the relative amounts of calmodulin in the particulate and soluble fractions in animal cells depends on the presence of calcium or EGTA (Vanaman *et al.*, 1976; Sobue *et al.*, 1979; Drabikowski *et al.*, 1978; Kakiuchi *et al.*, 1978). Calmodulin is usually high in the particulate fraction in the presence of calcium, apparently due to binding to calmodulin-binding proteins attached to membranes. Although high (2M) salt concentrations have not been found to release calmodulin from the particulate fraction of brain (Kakiuchi *et al.*, 1978), it seems possible that high salt concentrations may increase extractability of calmodulin from pea tissue if it is bound to proteins that are loosely bound to membranes. As it is known that calmodulin does occur in organelles and in the microsomal fraction of plant cells (Muto, 1982), and an examination of a plasma membrane fraction of pea root (obtained from Dr.A.Hetherington) confirmed that it was found in the membrane fraction of pea root tissue, calmodulin may have remained in the particulate fraction during extraction from pea root. It had also been noted that it was necessary to include a high salt concentration and EGTA in the initial homogenisation buffer in the extraction of calmodulin-dependent NAD kinase by the procedure of Anderson and Cormier as a buffer of Tris and PVPP was not adequate to extract this calmodulin-dependent enzyme. Calmodulin could also be detected in this crude extract although it was not detectable in the absence of EGTA and salt in the buffer. This result reinforced the possibility that calmodulin-binding proteins might also be present in membrane fractions or in organelles in pea tissue, and that stronger extraction conditions than those described by Anderson and Cormier (1978) might be required to release calmodulin from them.

Although the modification of the procedure of Charbonneau and Cormier to include heat treatment early in the purification

might have released some of the calmodulin bound to membranes (Yamanaka and Kelly, 1981), particularly as heating was carried out in the presence of EGTA, the initial solubilisation might have been low as salt and EGTA were not included in the homogenisation buffer.

I therefore compared the amount of calmodulin from pea shoots detectable by NAD kinase assay using six different extraction procedures. Three of these employed the initial extraction procedure of Anderson and Cormier (1978) in 20mM Tris, 2% PVPP pH 8. After centrifugation the supernatant was taken to 50% saturation with ammonium sulphate. This solution was then treated in one of the following ways:

Method 4:

a) The solution was centrifuged for 20 minutes at 12,000G and the pellet resuspended in 20mM Tris pH 8, 1mM EGTA and dialysed against this buffer for 18 hours. The dialysate was heated to 100°C for 2 minutes, then centrifuged for 30 minutes at 12,000G to remove precipitated material. The supernatant was dialysed against 20mM Tris pH 8 for 12 hours.

b) The solution was centrifuged at 12,000G for 20 minutes and the supernatant taken to pH 4, with 1M H_2SO_4 in 50% ammonium sulphate. After stirring the solution for two hours, the solution was centrifuged for 30 minutes at 12,000G. The pellet was resuspended in 1mM EGTA, 20mM Tris pH 8 and was dialysed, heated and dialysed against Tris pH 8 as described for a).

c) The ammonium sulphate solution was taken directly to pH 4 with 1M H_2SO_4 in 50% ammonium sulphate and stirred for two hours. The solution was then centrifuged for 30 minutes at 12,000G and the pellet resuspended in 1mM EGTA, 20mM Tris pH 8. Dialysis, heat treatment and final dialysis against Tris pH 8 were carried out as described for a).

These procedures were repeated using 1M KCl, 1mM EGTA, 50mM Tris pH 7.4, 2mM $MgCl_2$, 2.5% PVPP as the initial homogenisation buffer, (Methods 5a-c). The results are shown in table 3:2:1.

The results indicated that the neutral ammonium sulphate fraction used by Anderson and Cormier contained little extractable calmodulin. Calmodulin was found to be present in the ammonium

sulphate supernatant at neutral pH, and could be recovered by isoelectric precipitation at pH 4 to yield six times that obtained from the ammonium sulphate pellet. However, taking the entire ammonium sulphate solution to pH 4 extracted a considerably greater amount, approximately sixty five times as much calmodulin as from the original ammonium sulphate pellet.

Addition of salt and EGTA to the extraction medium made little difference to extraction of calmodulin from the ammonium sulphate pellet or supernatant: however, the yield was increased a hundredfold over that obtained by the original method, when the 50% ammonium sulphate solution was taken to pH 4. This method yielded 23µg calmodulin/g pea shoot, equivalent to 0.4% of total protein when assayed by NAD kinase activation calibrated with bovine brain calmodulin. The yield was higher than that obtained using the same procedure with the extraction buffer of Anderson and Cormier, indicating that some calmodulin may indeed have remained attached to membranes, despite heat treatment of the sample.

This comparison was repeated with pea root tissue, and the results were found to be very similar. Increasing the ammonium sulphate concentration to 60% was found to increase the yield by a further 25% (Method 5d). Further purification by affinity chromatography was not included as the losses incurred were too high (Charbonneau and Cormier, 1979). A comparison of all extraction methods is given in table 3:2:1.

e) Final extraction procedure

The final procedure for extraction of calmodulin, method 5d, was designed to obtain a highly purified sample without the use of affinity chromatography which involved substantial losses, and to minimise interference from calmodulin-binding proteins throughout extraction. The procedure included homogenisation in a high salt and EGTA containing buffer, isoelectric precipitation in 60% ammonium sulphate, and heat treatment following dialysis against EGTA. Details are provided in chapter 2 section 2 H i c I. Although not demonstrated conclusively to be calmodulin, the sample displayed many of the characteristics of calmodulin including heat stability, acidic

Table 3:2:1. Comparison of yields of calmodulin from pea tissues using different extraction procedures

Method of extraction and tissue used	µg calmodulin/g fresh weight		calmodulin as a % protein	
	1	2	1	2
Method 1. Anderson and Cormier (1978) Crude homogenate Root tissue 0-10mm	0		0	
Method 2. Charbonneau & Cormier (1979) Shoot tissue	0	0	0	0
Method 2. Charbonneau & Cormier (1979) Peanut seed	6.8[3]		0.002%[3]	
Method 3. Charbonneau & Cormier (1979) (modified)				
Shoot	0.2	8	0.003%	0.12%
Root 0-2mm	0	13,000	0	23%
Root 8-10mm	0	609	0	10%
Method 3. Charbonneau & Cormier (1979) (Modified. Prior to affinity chromatography) Root 0-10mm	7		0.01%	
Method 4. Based on Anderson & Cormier (1978) Homogenised in 20mM Tris pH8, 2.5% PVPP. Shoot tissue Modifications:				
a) 50% ammonium sulphate pellet	0.25		0.003%	
b) 50% ammonium sulphate supernatant precipitated at pH4	1.43		0.18%	
c) 50% ammonium sulphate pH4 pellet	16.5		0.21%	

Method 5.

Based on Anderson *et al.*(1980)

Homogenisation buffer included
1mM EGTA, 1M KCl.

Shoot tissue		
Modifications:		
a) 50% ammonium sulphate pellet	0.22	0.0036%
b) 50% ammonium sulphate supernatant precipitated at pH4	0.28	0.005%
c) 50% ammonium sulphate pH4 pellet	23	0.38%
Root tissue 0-10mm		
a) 50% ammonium sulphate pellet	2.5	0.005%
b) 50% ammonium sulphate supernatant precipitated at pH4	8	0.015%
c) 50% ammonium sulphate pH4 pellet	67	0.12%
d) 60% ammonium sulphate pH4 pellet	84	0.15%

Caldwell & Haug (1981a)		
Bovine brain (acetone powder)	220	0.74%[4]
Bovine brain	74.6	0.25%[4]

1. Estimated by activation of NAD kinase using bovine brain calmodulin as a standard.
2. Estimated by A_{276} values.
3. Estimated by bovine phosphodiesterase activation.
4. Estimated on the assumption that protein constitues 3% of the fresh weight of brain (Watterson *et al.* 1976).

isoelectric point, calcium-dependent activation of phosphodiesterase and NAD kinase (figure 3:2:6), and phenothiazine inhibition of enzyme activation (figure 3:2:6). Properties are summarised in table 3:2:2. This sample will henceforth be referred to as calmodulin. Two-six polypeptides were observed on two-dimensional polyacrylamide gels of the samples. However, the samples differed from bovine brain calmodulin in that no polypeptide of similar molecular weight and pI' value was observed on the gels, and no polypeptide was observed to undergo a calcium-dependent mobility shift during electrophoresis (figure 3:2:7). This will be further discussed in section iii b.

The final extraction procedure was a considerable improvement over the original. By this procedure, up to five hundred times as much calmodulin as a percent of total protein was extracted from pea roots as that from peanut seed by the method of Charbonneau and Cormier depending on the root zone, while no calmodulin had been detected from pea seedlings using the original extraction procedure of Charbonneau and Cormier (1979). From these figures, it would appear that calmodulin in the pea root is present at the same level of concentration as in non-neurosecretory cells in animals on a percent of cellular protein basis (Watterson *et al.* 1980).

(iii) Modification of calmodulin during extraction

It was very important when comparing the concentration of calmodulin from different tissues to know whether differential losses, modification, or extractability were occurring, and to know whether the preparations contained any component that influenced either calmodulin or the enzyme to be assayed. This was particularly important as the results from polyacrylamide gel electrophoresis of the highly purified sample of calmodulin indicated that it might be modified during extraction, or that it differed in some aspects from bovine calmodulin.

a) Modification of activating ability

I therefore compared the activating abilities of samples

Table 3:2:2. Comparison of properties of calmodulin isolated from bovine brain and from pea root

Property	Source of calmodulin: Bovine brain[1]	Pea root[2]	Pea root[3]
Molecular weight (from SDS-PAGE):			
No control of Ca^{2+} in electrophoresis	17,000		18,000
Ca^{2+}-dependent mobility shift in SDS-PAGE to a faster migration rate:	+		+
+ Ca^{2+} in sample buffer	15,500 (17,000) (18,500)		16,000
+ EGTA in sample buffer	20,500 (22,000)		18,000
+ EGTA in sample and electrophoresis buffers	21,500		21,000 (16,000) (18,000)
+ EGTA in first dimension + $CaCl_2$ in second dimension (2-D PAGE)[2]	18,000 (20,000)		18,000
+ $CaCl_2$ in first dimension + EGTA in second dimension (2-D PAGE)	18,000 (20,000)		18,000
Ca^{2+}-dependent mobility shift in non-denaturing gels to a faster migration rate	+		+
Isoelectric point	pH 3.9		pH 3.9
Isoelectric precipitation from solution	pH 4	pH 4	
Heat stable	+	+	
Ca^{2+}-dependent activation of calmodulin-dependent NAD kinase	+	+	
Ca^{2+}-dependent activation of calmodulin-dependent bovine heart phosphodiesterase	+	+	
Phenothiazine inhibition of NAD kinase activation	+	+	
Phenothiazine inhibition of phosphodiesterase activation	+	+	
Ca^{2+}-dependent binding to a phenothiazine affinity column	+	+	

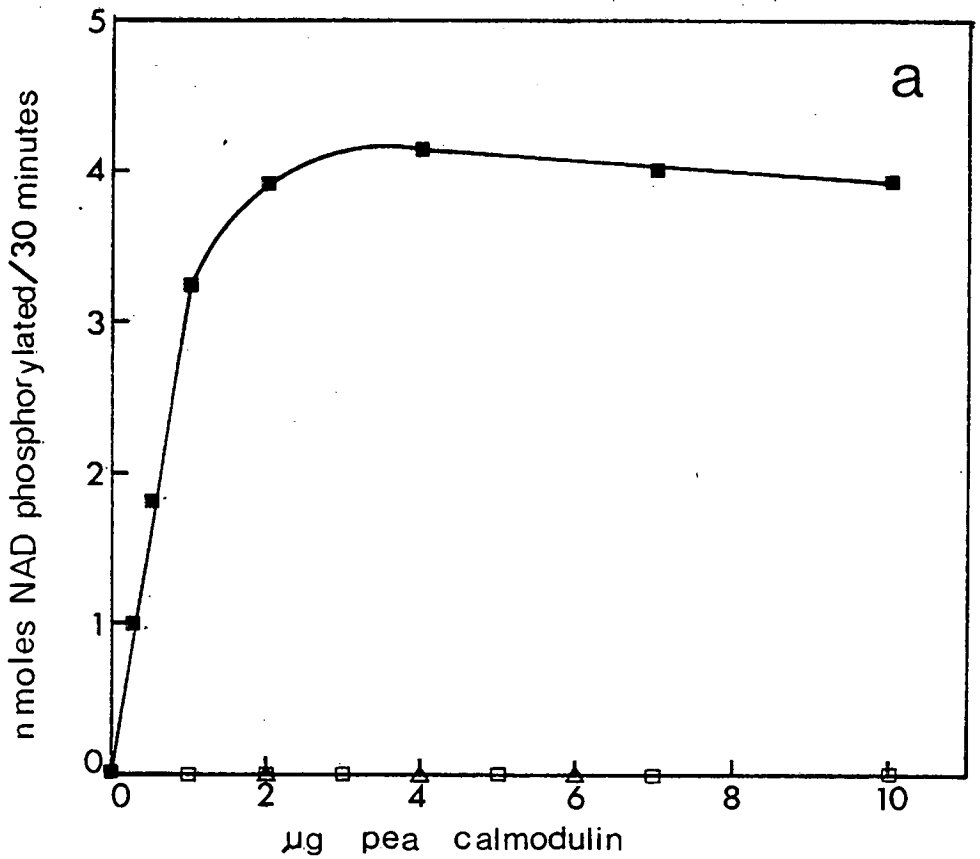
1. Bovine brain calmodulin isolated by the procedure of Caldwell and Haug (1981a).
2. Pea root calmodulin isolated by method 5d as described in section 2 B ii e.
3. Pea root proteins separated from a crude homogenate by two-dimensional polyacrylamide gel electrophoresis.

Figure 3:2:6. Calcium-dependent activation of NAD kinase and phosphodiesterase by pea root calmodulin

a) Pea root calmodulin was purified by method 5d as described in chapter 3 section 2 B ii e, and was used to activate calmodulin-dependent NAD kinase in the presence of calcium (——■——). Calmodulin-dependent NAD kinase was obtained from pea shoot by the method of Anderson *et al.* (1980), and assayed by a modification of the procedure of Muto and Miyachi (1977) as described in chapter 2 section 2 I. The concentration of calmodulin was estimated by comparison of activating ability with that of known quantities of bovine brain calmodulin. Inhibition of enzyme activation was carried out using 2mM EGTA (——□——) or 80μM trifluoperazine (——△——) in the incubation medium. The results are expressed as nmoles NADP phosphorylated from NAD per 30 minute incubation.

b) Pea calmodulin purified and estimated as described in figure a) was used to activate calmodulin-dependent bovine heart 3':5'-cAMP phosphodiesterase in the presence of calcium (——■——). Assays were carried out according to the method of Cheung (1971). Inhibition of enzyme activation was carried out using 2mM EGTA (——□——) or 80μM trifluoperazine (——△——) in the incubation medium. The results are expressed as inorganic phosphorus released from 5' AMP produced by 5'-nucleotidase activity. 5' AMP is produced during the assay as a result of cAMP hydrolysis by phosphodiesterase activity.

Fig. 3:2:6.


a
n moles NAD phosphorylated/30 minutes
0
1
2
3
4
5
0
2
4
6
8
10
µg pea calmodulin

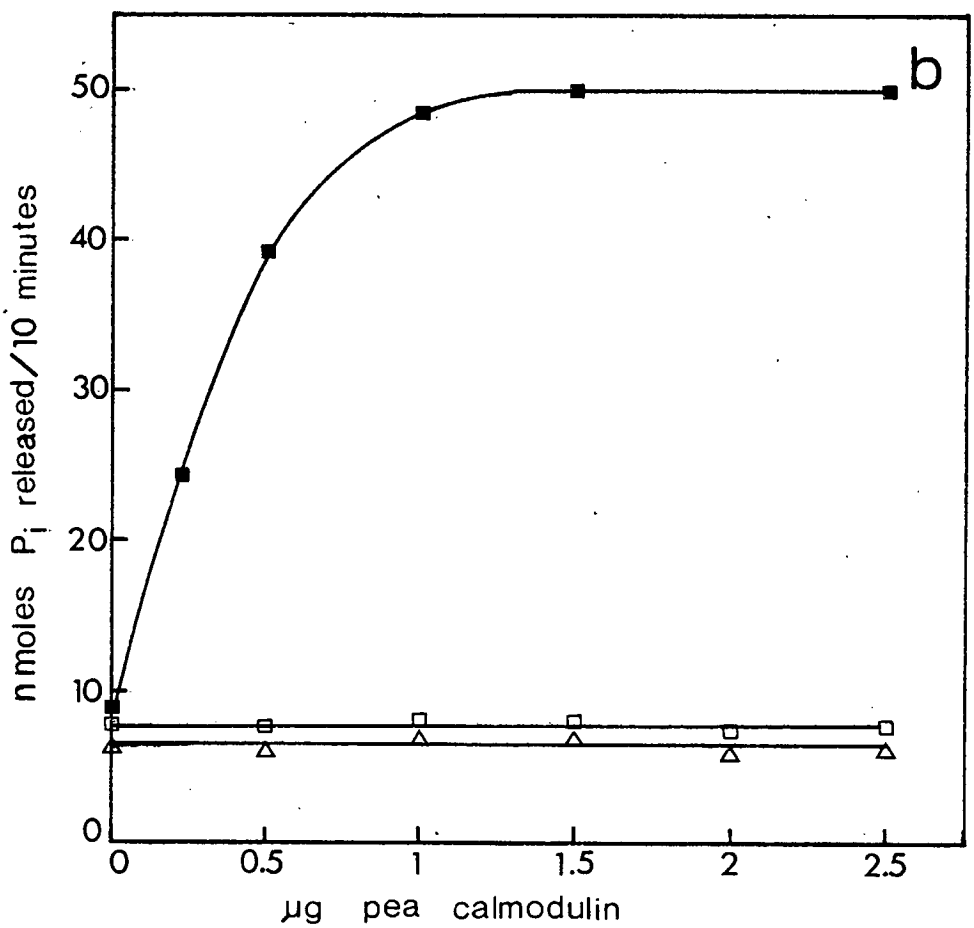

b
n moles P_i released/10 minutes
0
10
20
30
40
50
0
0.5
1
1.5
2
2.5
µg pea calmodulin

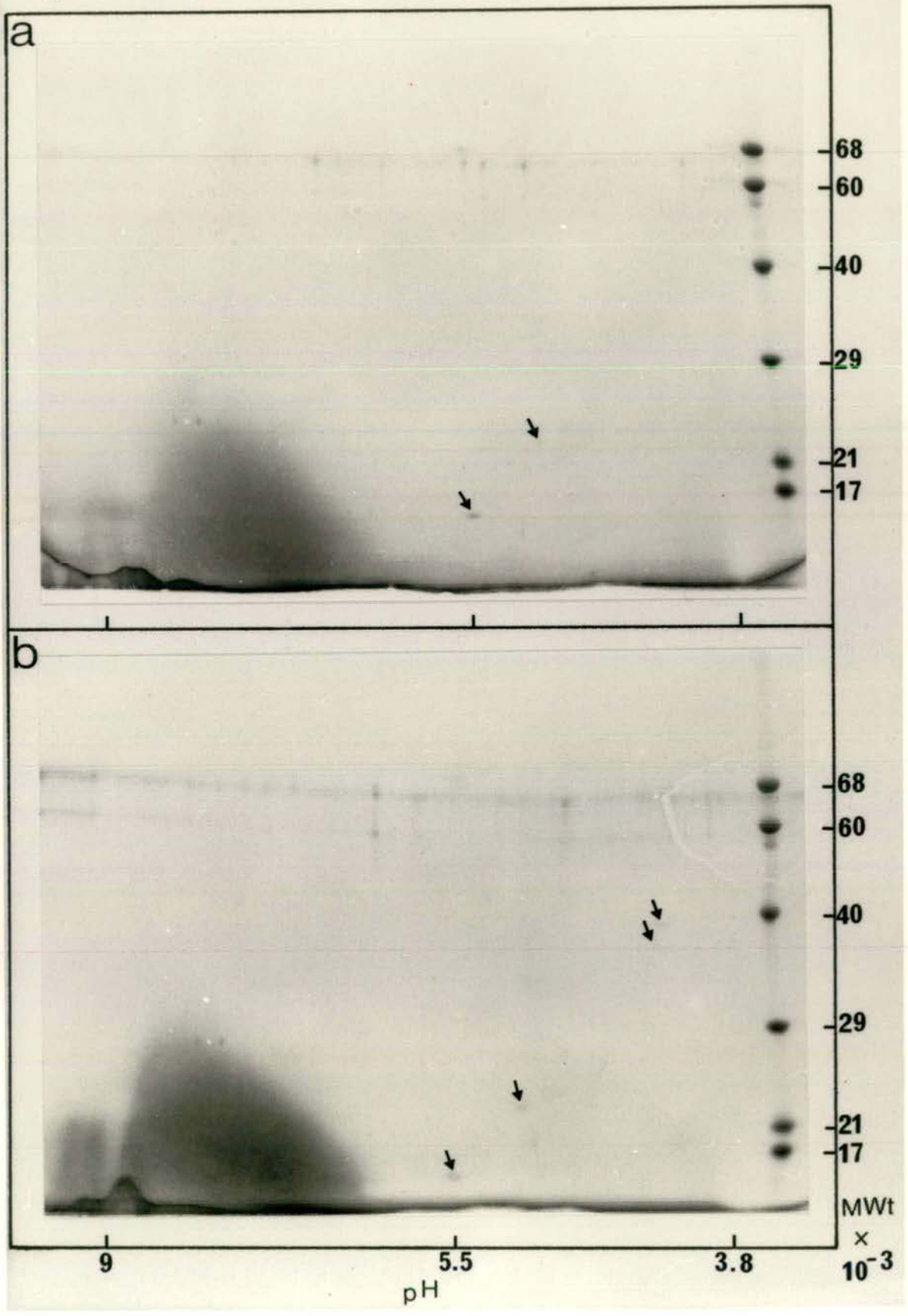
Figure 3:2:7. Two-dimensional polyacrylamide gel electrophoresis of partially purified pea root calmodulin

Calmodulin was extracted from pea roots by method 5d in the text. Protein was electrophoresed by NEPHGE in the 1st dimension and by SDS-PAGE in the 2nd dimension, as described in chapter 2 section 2 G iii.

a) Partially purified calmodulin was obtained from pea root tissue 5-6.25mm from the apex.

b) Partially purified calmodulin was obtained from pea root tissue 3.75-5mm from the apex.

Fig. 3:2:7.



of calmodulin extracted from 10mm sections of pea root homogenised with and without a known quantity of purified bovine brain calmodulin of known activating ability. NAD kinase activation was increased to the extent expected in the control containing added calmodulin. No losses or inhibition seemed to have occurred during extraction, and any modifications did not appear to alter its activating ability *in vitro*. However, it is possible that endogenous calmodulin is more accessible or more susceptible to inhibitory or modifying factors.

Promoters of NAD kinase activity other than calmodulin did not appear to be present, as no activation occurred in the presence of trifluoperazine or EGTA. However, a slight inhibition of the NAD kinase assay with increasing amount of the highly purified sample of pea root calmodulin occurred in extracts from some zones of the root. This was found to be due to inhibition of NAD kinase activity, not of G6Pdh activity. However, the effect was slight, and as there was no correlation between concentration of calmodulin and degree of inhibitory effect, it would appear that inhibition or promotion of the NAD kinase assay were not significant factors influencing the enzymic assay of calmodulin.

As modifications of the extraction procedure had produced such different yields of calmodulin, it was possible that the extractability might vary considerably between different tissues. It was not possible to obtain even a rough estimate of the proportion of calmodulin extracted as crude homogenates of calmodulin displayed very little activating ability, presumably at least partly as a result of interference by calmodulin-binding proteins. Despite the use of high salt concentrations and EGTA during homogenisation, it is possible that differential extractability may occur within the root apex.

b) Modification of mobility on polyacrylamide gels

The samples of calmodulin obtained from different sections of the root were observed on two-dimensional polyacrylamide gels. However, despite loading of up to 75 µg calmodulin as estimated by NAD kinase assay calibrated with bovine brain calmodulin, no polypeptides of the molecular weight and isoelectric point of

calmodulin were detected.

Two to six polypeptides were detected on the gels depending on the zone from which the sample was obtained. The major polypeptide on these gels migrated with a mobility consistent with a molecular weight of 16,000 daltons in SDS, but migrated to pH 5.5 in the isoelectric focusing dimension (shown in figure 3:2:7). As it was possible that modification of charge had occurred, the samples were then electrophoresed in the presence of either calcium or EGTA in two-dimensional and one-dimensional gels. However, none of the polypeptides on the gel displayed a mobility shift. As slight differences in pI in plant and bovine calmodulins are to be expected, while phosphorylation of calmodulin results in a lower pI (Plancke and Lazarides, 1983), NEPHGE gels were then electrophoresed from the basic end to allow acidic proteins to lead in the separation, and to alter the pH gradient. The time of electrophoresis was also varied between 400 and 4000 Vhours to ascertain whether a polypeptide might be lost from the gel or whether calmodulin might require a longer time to reach its isoelectric point. However, no additional polypeptides were detected, and the polypeptide migrating to pH 5.5 remained at the same position in the pH gradient.

It is not clear whether this anomalous result is due to the presence of a calmodulin-like protein in pea, to modification of calmodulin during extraction, or to a difference in specific activity of bovine and pea calmodulins.

It is possible that a calmodulin-like protein was isolated by the procedure, as such proteins have been found in plants (Schleicher *et al.*, 1983; Van Eldik *et al.*, 1980b, 1980c). However, these proteins have isoelectric points similar to calmodulin, with pI values 0.3 units higher, and a calmodulin-like protein from *Chlamydomonas* flagella displays a small mobility shift in SDS polyacrylamide gels depending on the presence of calcium (Van Eldik *et al.*, 1980b) and does not activate calmodulin-dependent enzymes (Van Eldik *et al.*, 1980b, 1980c). None of the polypeptides isolated from pea root have properties similar to these calmodulin-like proteins.

Calmodulin may have been present in the solution, but in too low a concentration to be detected on polyacrylamide gels.

This is supported by the reports indicating that plant calmodulin is 7-fold more effective in activating plant NAD kinase than is calmodulin from animal sources, which would result in the over-estimation of pea calmodulin when bovine calmodulin is used as a standard.

It is also possible that calmodulin was altered during extraction to modify both its apparent isoelectric point and its capacity to bind calcium in SDS. Heat treatment, for example, could modify its conformation in 9M urea, its surface charge, or calcium-binding properties. It has been found that although the molecule can normally withstand temperatures of 100°C for 5 minutes, that extensive boiling for long periods may substantially reduce its biological activity (Beale *et al.*, 1977), and there are some instances where significant loss of activity is found after only 3 minutes at 100°C (Klee, 1977). Rapid loss of activity is possibly due to trapping of calmodulin in large precipitates of denatured protein when relatively impure extracts are heated, especially if heated in the presence of calcium (Klee, 1977). However, loss of activity due to heating crude homogenates in the presence of EGTA occurs with a half life of 7 minutes (Beale *et al.*, 1977) indicating that the protein is not completely heat stable.

It is known that calmodulin undergoes a reversible change in conformation on heating. Nuclear magnetic resonance studies indicate that the molecule appears to become almost fully denatured at 80°C in the absence of calcium as the spectrum at this temperature is close to that predicted for a random coil. On cooling, the molecule returns to its original conformation. Calcium stabilises calmodulin against heat-dependent change in conformation as indicated by a far less extensive change in the spectrum on heating fully calcium bound calmodulin (Niemczura *et al.*, 1982). In the purification procedure for pea calmodulin I heated samples in the presence of EGTA to avoid trapping of calmodulin during denaturation of calmodulin-binding proteins; however, it is possible that an irreversible change in conformation occurred at this stage due to inadequate stabilising of calmodulin to heat treatment. As it has also been found by Burgess *et al.* (1980) that heating alters the number of bands of calmodulin on

polyacrylamide gels, which is dependent on calcium binding, it also appears possible that the capacity for binding calcium in the presence of denaturing agents may be altered by heating. Heat treatment may therefore not only have altered the conformation of calmodulin, but also may have altered its capacity to bind calcium in the presence of SDS. This would result in atypical behaviour of the molecule on polyacrylamide gels, despite the retention of biological activity.

Another type of modification has been observed by Esnouf *et al.* (1980). They noted that the nuclear magnetic resonance and ultra violet spectra of calmodulins prepared under different conditions were significantly different. Two major types were observed. One of these did not undergo a calcium-dependent mobility shift in polyacrylamide gels, and the isoelectric focusing pattern was quite different from the other type. Apparently the type displaying anomalous behaviour had undergone limited proteolysis from the C-terminus, eliminating the fourth calcium-binding site. Although these fragments were unable to activate phosphodiesterase, Charbonneau *et al.* (1980) have observed that low molecular weight fragments found in calmodulin preparations from plants that had many characteristics of calmodulin, activated NAD kinase with a thirty-fold difference in activating ability to calmodulin. These fragments were suggested to be fragments of calmodulin. It therefore also seems possible that limited proteolysis may have occurred in purification of pea calmodulin, reducing specific activity drastically, with one or more of the polypeptides on the gels possibly being calmodulin. As activity of bovine calmodulin was not affected by this extraction procedure, it is possible that bovine and pea calmodulins have different stabilities towards pea root proteases. The possibility of modification of calmodulin during extraction to produce anomalous migration could be investigated by comparing gels of bovine brain calmodulin homogenised with pea root protein and taken through the extraction procedure, with gels of untreated bovine brain calmodulin.

(iv) Summary

Several problems were encountered in the purification of

calmodulin from pea tissue. It was found that calmodulin could not be extracted from pea root or shoot tissue without providing conditions that separate calmodulin from calmodulin-binding proteins including those present in membranes, and without providing conditions that will remove these proteins before affinity chromatography. It was also found that 50% ammonium sulphate at neutral pH values was not sufficient to precipitate calmodulin, and isoelectric precipitation had to be included. The final procedure included homogenisation in a buffer containing a high salt concentration and EGTA, precipitation by 60% ammonium sulphate at pH4, and heating for 2 minutes at 100°C after dialysis against EGTA. The final procedure (method 5d) extracted up to five hundred times as much calmodulin from pea root tissue and two hundred times as much from shoot tissue as that obtained from peanut seed by the method of Charbonneau and Cormier. Extractable calmodulin was 0.4% of total cellular protein from shoot tissue as assayed by NAD kinase activation, thus being similar to the concentration of extractable calmodulin in non-neurosecretory animal cells. A comparison of the characteristics of bovine and pea calmodulins is provided in table 3:2:2.

The sample of protein obtained by this procedure displayed several of the characteristics of calmodulin including heat stability, calcium-dependent activation of NAD kinase and phosphodiesterase, inhibition of enzyme activation by low concentrations of phenothiazines, calcium-dependent binding to a phenothiazine affinity column, and acidic isoelectric point. However, two - six polypeptides were observed on two-dimensional gels, none displaying the characteristic behaviour of calmodulin on polyacrylamide gels. This could be due to the presence of a calmodulin-like protein in pea root. However, it is more likely to be the result of an overestimation of calmodulin due to different capacities of bovine and pea calmodulins to activate the enzymes used in assaying concentration; or to be a result of proteolysis resulting in reduction of biological activity and alteration in electrophoretic properties tending to underestimate calmodulin; or to some other modification during extraction, possibly altering conformation or affecting calcium-binding

properties under denaturing conditions, producing anomalous migration on polyacrylamide gels although not necessarily affecting biological activity.

It was therefore very important to identify calmodulin on polyacrylamide gels or to identify calmodulin in the samples by a more direct method than those already employed. As the sample was not pure it was not possible to analyse the amino acid composition, or to carry out peptide mapping by limited proteolysis such as that described by Cleveland *et al.* (1977). Antibody techniques were not considered to be useful methods to investigate calmodulin due to severe problems in obtaining satisfactory antibodies (Wallace and Cheung, 1979; Chafouleas *et al.*, 1979; Dedman *et al.*, 1978; Jones *et al.*, 1980; Welsh *et al.*, 1978), and to cross-reaction of the antisera with proteins other than calmodulin such as a calmodulin-like protein from *Chlamydomonas reinhardtii* (Van Eldik *et al.*, 1980b; van Eldik and Watterson, 1980) and *Dictyostelium discoideum* (Chafouleas *et al.*, 1979). However, I felt that it would be useful to attempt to identify the position of calmodulin on polyacrylamide gels by affinity labelling with radioactive trifluoperazine. This is briefly described in section C ii b.

C. Estimation of calmodulin during root development

(i) Estimation of calmodulin by enzyme activating ability

Samples of calmodulin were purified almost to homogeneity as analysed by two-dimensional polyacrylamide gel electrophoresis. As indicated in the previous sections the activating ability of the protein did not appear to be modified by extraction, nor did there appear to be any component present in the sample that modified the NAD kinase assay to any significant extent. Although the protein possessed many of the properties of calmodulin it was not demonstrated conclusively to be calmodulin. However, it appeared to be functionally identical to calmodulin, as summarised in table 3:2:2. Further attempts to identify calmodulin indirectly in this sample were made by affinity binding of radioactively labelled trifluoperazine.

Calmodulin-like activity in this sample was assayed by NAD kinase activating ability and compared to activation by known amounts of bovine brain calmodulin. It is possible that calmodulin-like activity was overestimated by this method as described previously.

As the purified activator could not be identified on polyacrylamide gels, calmodulin-like *in vitro* biological activity could not be directly compared to the concentration of calmodulin estimated by gel electrophoresis. However, an estimation of the relative amounts of calmodulin present during differentiation in the root apex was obtained from two-dimensional polyacrylamide gel electrophoresis of protein from crude extracts, and this was compared to the estimates obtained by NAD kinase activation.

(ii) Estimation of calmodulin by polyacrylamide gel electrophoresis

Purified pea calmodulin could not be identified on polyacrylamide gels. Calmodulin was therefore tentatively identified on two-dimensional gels obtained from crude extracts of pea root and estimates of calmodulin concentration during differentiation were obtained by observing this polypeptide.

a) Identification of calmodulin from crude extracts on two-dimensional polyacrylamide gels

When pea roots were homogenised in 9.5M urea and electrophoresed as described in chapter 2, a polypeptide migrating to a pH of 3.9 and molecular weight of 16,000 daltons was observed on two-dimensional NEPHGE gels. This protein was lost to the anode during isoelectric focusing as the pH gradient altered to begin above pH 4. It was found that the electrophoretic mobility and number of spots observed of this polypeptide in the SDS dimension varied according to the presence of calcium or EGTA in the sample and electrophoresis buffers as shown in figure 3:2:8. In the presence of calcium a band at pH 3.9 migrated to a position corresponding to about 16,000 daltons. When EGTA was added to the sample and electrophoresis buffers this band disappeared, and a major band was observed at 21,000 daltons, and two minor bands at 16 and 18,000. However, if calcium was added in the NEPHGE dimension and EGTA in the SDS dimension, or vice versa,

Figure 3:2:8. Two-dimensional polyacrylamide gel electrophoresis of pea root proteins demonstrating a calcium-dependent mobility shift of a calmodulin-like protein

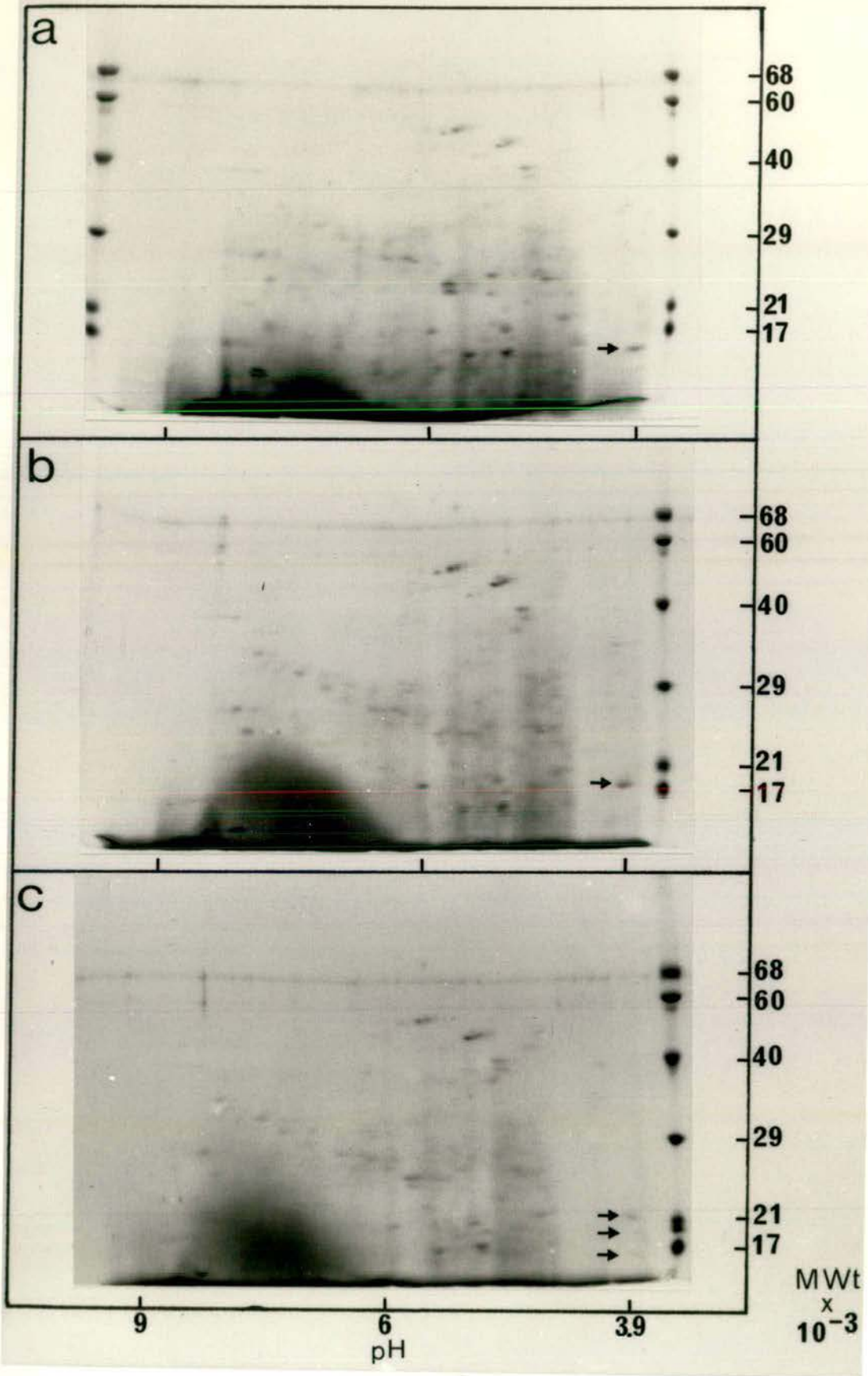
Sections of the apical 2mm of pea root were homogenised in buffer containing 9.5M urea. Proteins were separated by NEPHGE in the 1st dimension and by SDS-PAGE in the 2nd dimension as described in chapter 2 section 2 G iii.

a) 10mM $CaCl_2$ was added to the homogenisation buffer.

b) 10mM $CaCl_2$ was added to the homogenisation buffer prior to electrophoresis in the 1st dimension. 1.5mM EGTA was added to the SDS electrophoresis buffer in the 2nd dimension.

c) 15mM EGTA was added to the homogenisation buffer prior to electrophoresis in the 1st dimension, and 1.5mM EGTA was added to the SDS electrophoresis buffer in the 2nd dimension.

Fig. 3:2:8.



a band at 18,000 daltons was observed. This protein migrated with a similar mobility to bovine brain calmodulin in two-dimensional gels; both migrating to pH 3.9 in the first dimension, and with a difference in the SDS dimension of about 500 daltons in the presence of calcium (figure 3:2:9).

This behaviour is entirely consistent with the behaviour of calmodulin as described in section 2 A iii b. Plant calmodulins (Van Eldik *et al.*, 1980a, 1980c; Muto, 1983) have been found to migrate with a mobility equal to, or up to 1000 daltons faster than animal calmodulin in SDS gels depending on the presence of calcium, while it tends to undergo less of a mobility shift in EGTA than animal calmodulin. The amino acid composition of plant calmodulin is also similar to that of animal calmodulin, differing only in a few amino acid residues (Charbonneau *et al.*, 1980) and they would therefore be expected to migrate to similar positions on urea-pH gradient gels. The few proteins other than calmodulin including calmodulin-like proteins that undergo a calcium-dependent mobility shift have a far smaller shift of 500-1000 daltons (Grand *et al.*, 1980; Van Eldik *et al.*, 1980b). As the putative calmodulin from pea root underwent a mobility shift of about 5,000 daltons, and as no other polypeptide underwent a mobility shift or migrated to a similar position on the gels, it is very likely that this protein is indeed calmodulin.

b) Modification of mobility of calmodulin from crude extracts on polyacrylamide gels

Due to the problems discussed in previous sections it is clear that the position and apparent heterogeneity of calmodulin on both isoelectric focusing and SDS gels may not bear relation to its isoelectric point or molecular weight. This is mainly dependent on the presence of calcium and of calmodulin-binding proteins.

It is known that calmodulin may remain bound to proteins in 6-8M urea (Head *et al.*, 1977; Grand *et al.*, 1980) and that these complexes may be only partially dissociated by removal of calcium (Grand and Perry, 1979; Grand *et al.*, 1979). It was therefore possible that such complexes remained intact in the presence of traces of calcium even in 9.5M urea in the first

dimension gel, but became dissociated during electrophoresis in the second dimension in the presence of SDS. The position to which calmodulin would migrate in the first dimension would then depend on the pI of the complex.

To investigate the possibility of interference by calmodulin-binding proteins in the isoelectric focusing dimension, I examined two-dimensional gels of proteins from crude extracts of pea roots and from a calmodulin-affinity column electrophoresed in the presence of calcium or EGTA.

Proteins eluted from a calmodulin-affinity column with EGTA did not display any binding to bovine calmodulin in two-dimensional gels as shown in figure 3:2:10, nor could I discern any difference in intensity or position of polypeptides in crude homogenates of pea root between calcium and EGTA run gels. Similarly, the apparent molecular weight and isoelectric point of bovine calmodulin were not altered when homogenised with pea roots (figure 3:2:9). As it was also noted that the intensity of staining of the putative calmodulin did not increase in the presence of EGTA as would be expected if such complexes had formed, it seems very unlikely that calmodulin-binding proteins are a significant source of artifact in two-dimensional polyacrylamide gels. This could be further investigated by binding ^{125}I-calmodulin to the gels (Carlin *et al.*, 1980) to identify calmodulin-binding proteins, and ^{3}H trifluoperazine or fluorescently-labelled antibodies to identify calmodulin.

The isoelectric point and molecular weight of bovine calmodulin did not appear to alter when it was homogenised with pea root tissue and subjected to two-dimensional electrophoresis as shown in figure 3:2:9. Modification of the isoelectric point and molecular weight of calmodulin does not, therefore, appear to occur with the extraction procedure employed.

A further possible source of error in the estimation of calmodulin by electrophoresis was variation in extractability of calmodulin in different root zones depending on its partitioning between soluble and membrane fractions. Compartmentation and association with membranes might be expected to vary within the root according to organelle development and presence of calmodulin-dependent membrane proteins. Although 9.5M urea will disrupt the membrane fraction substantially, not all

Figure 3:2:9. Two-dimensional polyacrylamide gel electrophoresis of pea root proteins homogenised with bovine brain calmodulin

Sections from the apical 2mm of pea root were homogenised with 4µg of bovine brain calmodulin obtained by the procedure of Caldwell and Haug (1981a) in the presence of 10mM $CaCl_2$. Proteins were separated by NEPHGE in the 1st dimension, and by SDS-PAGE in the 2nd dimension as described in chapter 2 section 2 G iii.

Bovine brain calmodulin is observed at 15,500 daltons. The putative pea calmodulin is observed at 16,000 daltons.

Fig. 3:2:9.

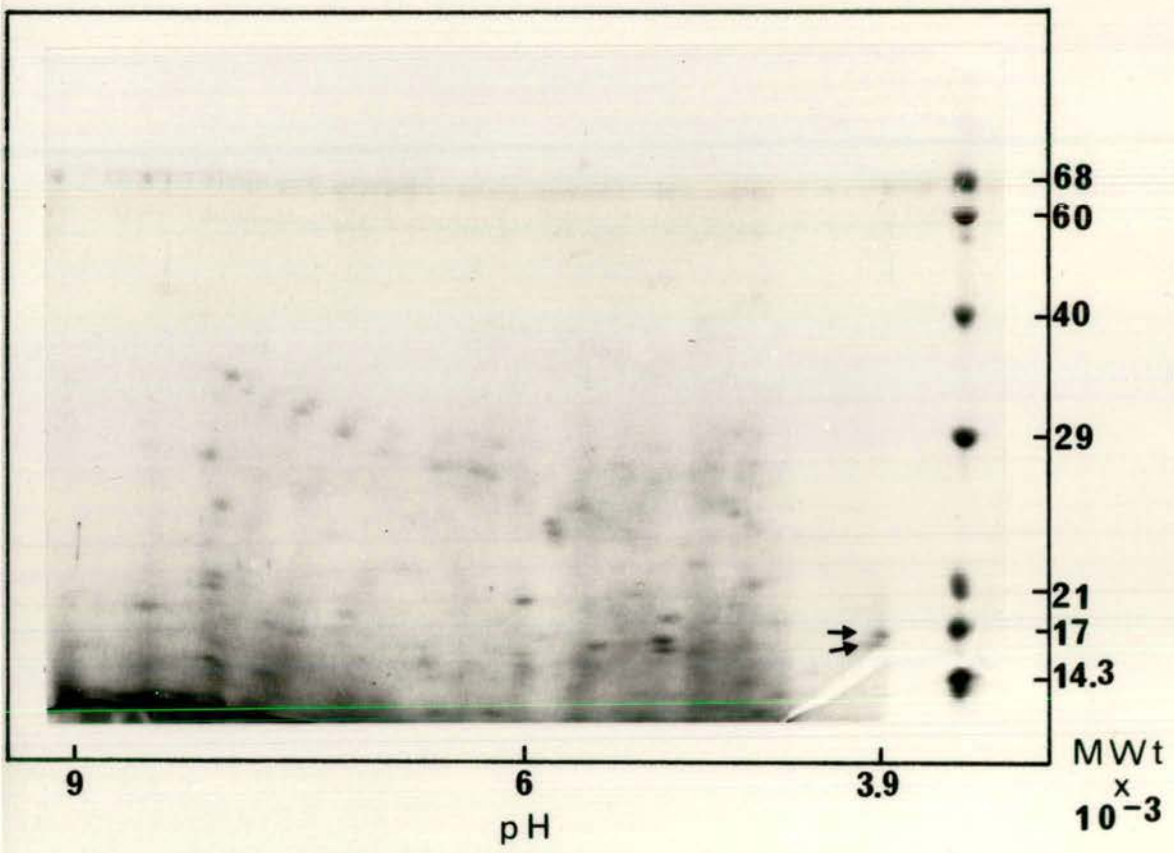


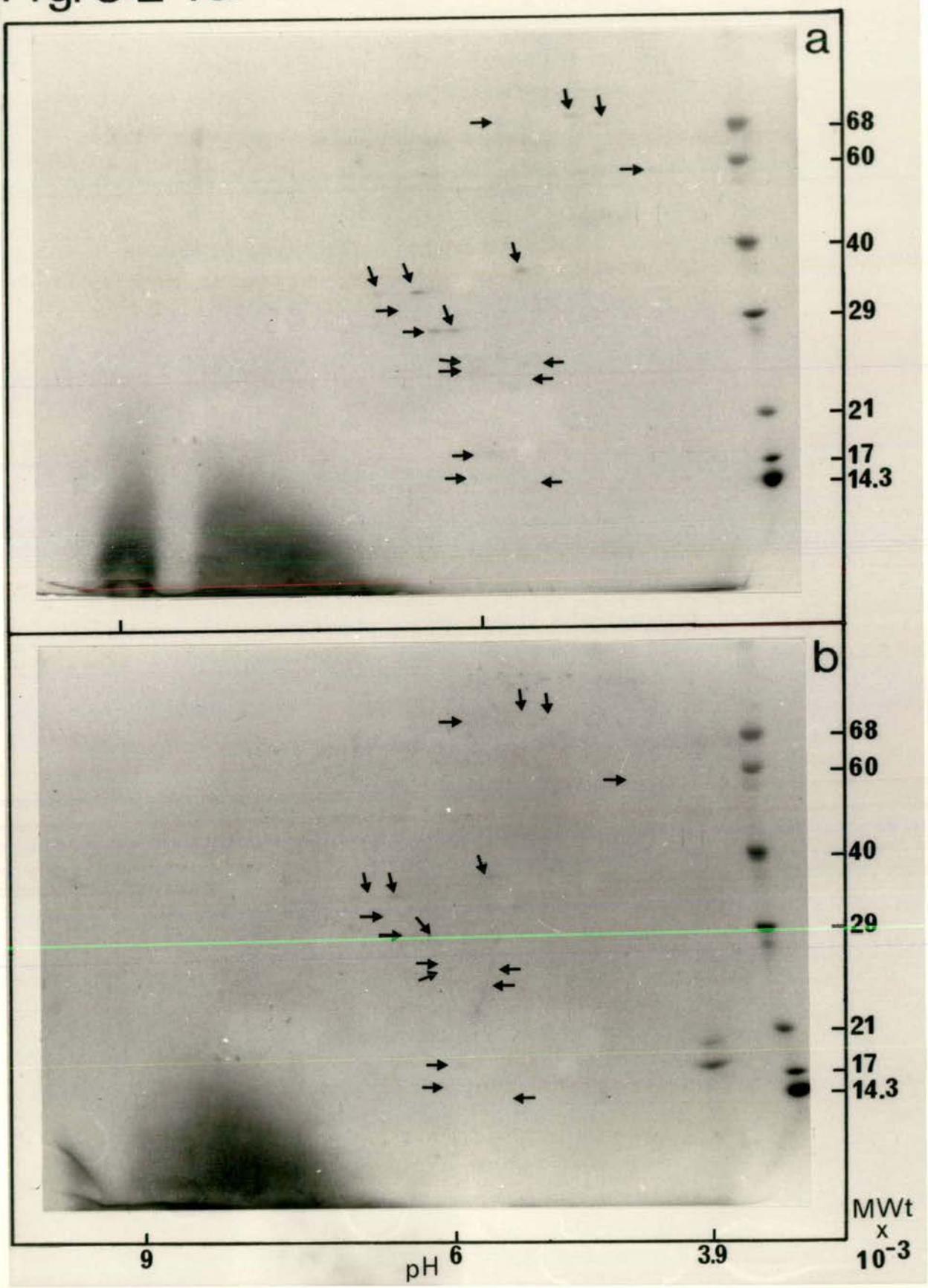
Figure 3:2:10. Two-dimensional polyacrylamide gel electrophoresis of pea root proteins binding to a calmodulin affinity column in a calcium-dependent manner

Proteins from the apical 10mm of pea root were extracted and applied to a calmodulin affinity column as described in chapter 2 section 2 H iii. Proteins binding in a calcium-independent manner were eluted with buffer containing 400mM KCl and 3mM $CaCl_2$. Proteins binding in a calcium-dependent manner were then eluted with EGTA. This sample was brought to 9.5M urea, and two volumes of buffer A (O'Farrell, 1975) were added. Proteins were then electrophoresed in the 1st dimension by NEPHGE, and in the 2nd dimension by SDS electrophoresis as described in chapter 2 section 2 G iii.

a) Two-dimensional polyacrylamide gel electrophoresis of pea root proteins binding to a calmodulin affinity column in a calcium-dependent manner. Samples contain 10mM $CaCl_2$.

b) Sample as for a) with the addition of 4µg of bovine brain calmodulin obtained by the method of Caldwell and Haug (1981a). 10mM $CaCl_2$ was added to the sample prior to electrophoresis.

Fig. 3:2:10.



proteins will have been solubilised, possibly including calmodulin-binding membrane proteins. However, as no increase in intensity of staining of the putative calmodulin occurred when crude extracts were homogenised in the presence of EGTA, binding to calmodulin-binding membrane proteins is probably not a significant factor interfering with extraction. Furthermore, as 90% of calmodulin is found in the soluble fraction of cells of wheat leaf in the presence of EGTA (Muto, 1982), it seems unlikely that differential extraction of calmodulin occurs between different zones of the root.

c) Identification of calmodulin on polyacrylamide gels by (^{3}H) trifluoperazine

It was clearly desirable for a number of reasons that both purified calmodulin obtained as described in section B ii e, and calmodulin from crude extracts as described in section C ii a should be identified by more direct means on polyacrylamide gels, as molecular weight, isoelectric point, calcium-binding properties, and heterogeneity on gels may be modified considerably by a number of conditions. It was felt that it might be possible to employ the specific binding characteristics of trifluoperazine to calmodulin to identify the protein on gels. To this end, calmodulin was immobilised on nitrocellulose paper by Western transfer after electrophoresis in SDS or non-denaturing gels, and incubated in radioactively labelled trifluoperazine under conditions favourable to specific binding of calmodulin. Following incubation, the nitrocellulose strips were washed, dried and fluorographed. The development of the procedure for identifying calmodulin on polyacrylamide gels by (^{3}H) trifluoperazine binding to calmodulin immobilised on nitrocellulose paper after Western transfer is described below.

I. Western transfer of pea root protein

Western transfer of protein from polyacrylamide gels was carried out according to Towbin *et al.*, (1979) with minor modifications. Transfer of protein was found to be poor under conditions described by Towbin *et al.*, and addition of 0.1% SDS to the transfer buffer was unfortunately required to permit

complete transfer of proteins from both SDS and non-denaturing gels, thus transferring proteins in a denatured state. All proteins appeared to migrate to the anode as no protein was detected on a nitrocellulose sheet placed on the cathodal side of the gel. The optimum time for transfer of proteins was found to be two hours at 56V and 400mA. Shorter transfer times resulted in incomplete transfer as assessed by Coomassie blue staining of the gel, and longer times resulted in loss of protein to the anode as a second nitrocellulose sheet placed to the anode side of the first began to accumulate protein while there was a corresponding decrease in these proteins on the sheet adjacent to the gel.

As described by Burnette (1981) the rate of transfer of protein was found to be dependent on MWt, lower MWt proteins migrating towards the anode more rapidly than higher MWt proteins. It was also found that lower MWt proteins, including calmodulin, appeared to be adsorbed more strongly and for a longer time to nitrocellulose sheet of a smaller pore size of 0.2μ than to nitrocellulose of a pore size of 0.45μ. 0.2μ pore size sheets were therefore used throughout.

Transfer from polyacrylamide gels, although complete, did not appear to be quantitative, as the relative intensities of staining of spots after Western transfer did not correspond to those on the original gels. Contrary to the finding of Erickson (1983) that inclusion of SDS in the transfer buffer with electrophoresis for 21 hours resulted in complete and quantitative transfer of proteins, increasing electrophoretic time to above two hours led to loss of protein from nitrocellulose to the anode. Altering the SDS concentration did not improve transfer. Howe *et al.* (1981) similarly noted that longer electrophoretic times may result in electrophoresis of proteins out of the nitrocellulose sheet towards the anode. Final conditions of Western transfer are described in chapter 2 section 2 G v a.

II. Detection of calmodulin by affinity binding to (^{3}H) trifluoperazine

Conditions of incubation of trifluoperazine with calmodulin

immobilised on nitrocellulose paper were aimed at maximising the degree of trifluoperazine bound to calmodulin without losing the specificity of binding.

It was noted that trifluoperazine binds non-specifically to low-affinity calcium-independent sites to many proteins (Weiss and Levin, 1978; Levin and Weiss, 1977), and that the degree of calcium-independent binding to a variety of proteins including calmodulin is near to that of calcium-dependent binding to calmodulin at 100μM trifluoperazine. Specificity of binding to calmodulin is therefore lost at concentrations of trifluoperazine of 100μM, irrespective of calcium concentration. One or two animal proteins have also been found to bind to trifluoperazine in similar amounts as calmodulin at levels of trifluoperazine over 10μM (Marshak *et al.*, 1981; Levin and Weiss, 1978). As the k_d of calcium-dependent binding of trifluoperazine to calmodulin is 1μM (Levin and Weiss, 1978), it was felt that concentrations of trifluoperazine between 1 and 10μM would permit relatively specific binding without substantial reduction in total amount of trifluoperazine bound.

Phenothiazines are light-sensitive, and all experiments with these drugs are normally performed in the dark. However, it was found (Prozialeck *et al.*, 1980; Cimino *et al.*, 1979) that irreversible binding of calmodulin to trifluoperazine occurred if incubation was carried out in the presence of calcium with irradiation by short wave ultra violet light at 254nm. There appeared to be one irreversible calcium-dependent binding site per molecule of calmodulin, while half-maximal binding occurred at 5μM trifluoperazine. Incubation of calmodulin immobilised on nitrocellulose with (^{3}H) trifluoperazine under ultra violet irradiation was therefore tested in an attempt to minimise any loss of trifluoperazine bound to calmodulin during washing procedures to remove excess unbound ^{3}H. Irreversible calcium-dependent binding was found to reach a maximum at one hour (Prozialeck *et al.*, 1980), however reversible binding in the presence of calcium has been found to reach equilibrium after six hours, and to remain constant for twenty hours in equilibrium dialysis (Levin and Weiss, 1977; Weiss *et al.*, 1980). A range of incubation times in the dark and under ultra

violet light were therefore tested between one and sixteen hours. Final incubation conditions are described in chapter 2 section 2 G v b.

As shown in figure 3:2:11, a radioactive band was seen on the fluorographs from nitrocellulose strips that were incubated in the presence of low concentrations of trifluoperazine to correspond to the position of the protein tentatively identified as bovine calmodulin on one-dimensional gels. Binding appeared to be calcium-dependent, and specific for calmodulin. Short incubation times with u.v. light of about 1 hour, or longer incubation periods of about 16 hours in the dark, were found to give maximum binding. Unfortunately the binding of calmodulin in the presence of calcium could not always be repeated. This was possibly as a result of denaturation of calmodulin by SDS, to the capacity of calmodulin for calcium binding after Western transfer, or to impurities that were known to be present in the sample of ^{3}H trifluoperazine. Due to lack of time the problem could not be investigated.

As the procedure was unreliable it was not used to investigate the position of calmodulin in complex mixtures or highly purified preparations of pea root proteins. Nevertheless, as specific calcium-dependent binding of trifluoperazine to the band corresponding to purified bovine brain calmodulin was demonstrated, it is a procedure worth investigating further.

d) Summary

A polypeptide of molecular weight 16,000 daltons and an isoelectric point of 3.9 from crude extracts of pea root underwent a calcium-dependent electrophoretic mobility shift consistent with the behaviour of calmodulin. The polypeptide was tentatively identified as calmodulin. A comparison of properties of this polypeptide with those of bovine calmodulin is provided in table 3:2:2. Bovine calmodulin did not appear to be altered by the extraction procedure for pea calmodulin, while complexing to calmodulin-binding proteins in the presence of 9.5M urea and differential extractability did not seem to be significant sources of error in estimating calmodulin in different areas within the root apex. The intensity of this

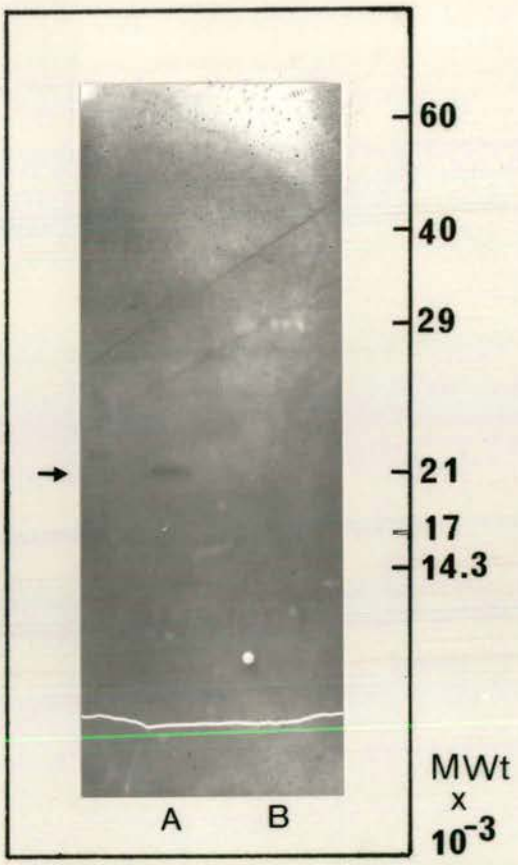
Figure 3:2:11. Fluorograph of a Western transfer of bovine brain calmodulin incubated with (^{3}H) trifluoperazine.

Bovine brain calmodulin obtained by the method of Caldwell and Haug (1981a) and a selection of molecular weight markers were electrophoresed by one-dimensional polyacrylamide gel electrophoresis as described in chapter 2 section 2 G i. Proteins were then transferred to nitrocellulose as described in chapter 2 section 2 G v a, and incubated with (^{3}H) trifluoperazine for 1 hour under u.v. light at 254nm as described in chapter 2 section 2 G v b. The nitrocellulose strip was then dried and fluorographed.

A. Bovine brain calmodulin.

B. Bovine serum albumin, catalase, aldolase, carbonic anhydrase, soybean trypsin inhibitor, myoglobin, lysozyme.

Fig. 3:2:11.



polypeptide on two-dimensional polyacrylamide gels during differentiation in the pea root apex was therefore compared to *in vitro* calmodulin-like activity during differentiation.

D Summary

Calmodulin was purified to homogeneity from bovine brain. This protein was used to calibrate calmodulin-dependent NAD kinase activity for the estimation of pea root calmodulin. It was also used to investigate the behaviour of calmodulin during its extraction from pea root both for NAD kinase activation and for polyacrylamide gel electrophoresis.

Although I have not been able to demonstrate conclusively the presence of calmodulin in pea root, the evidence indicates that calmodulin is indeed present in this tissue. This is indicated by the observation that a calmodulin-like polypeptide on two-dimensional gels displayed the unique property of calmodulin by undergoing a calcium-dependent electrophoretic mobility shift of several thousand daltons; and a highly purified protein preparation that had many of the properties of calmodulin including heat stability, acidic isoelectric point, and calcium-dependent binding to a phenothiazine affinity column appeared to be functionally identical to calmodulin, activating calmodulin-dependent pea NAD kinase and bovine heart phosphodiesterase in a calcium-dependent manner, with activation totally inhibited by low concentrations of trifluoperazine.

The NAD kinase activator was therefore purified almost to homogeneity from pea root tissue and its concentration estimated during root development by its ability to stimulate calmodulin-deficient NAD kinase. The estimates obtained for the concentration of this activator were compared to those obtained for the calmodulin-like protein on two-dimensional polyacrylamide gels.

3:3 DEVELOPMENT OF METHODS FOR THE EXTRACTION AND ESTIMATION OF NAD KINASE FROM PEA SEEDLINGS

Calmodulin-dependent NAD kinase was partially purified from pea seedlings for two purposes. Firstly, to provide an alternative means to polyacrylamide gel electrophoresis for detecting and quantitating very small amounts of calmodulin. Secondly, to investigate its distribution and activity in the root apex as a preliminary means of investigating the potential involvement of calmodulin in differentiation, and of investigating the significance of any change in concentration of calmodulin with differentiation.

A. Extraction and characterisation of NAD kinase

(i) The procedure of Anderson and Cormier (1978)

NAD kinase has been extracted from several plants and its activity found to be dependent on a low molecular weight, heat stable, protein activator (Muto and Miyachi, 1977). A partially purified NAD kinase preparation from pea seedlings was subsequently found by Anderson and Cormier (1978) to be activated 5-7 fold by calmodulin in a calcium-dependent manner, and the protein activator in plants demonstrated to be calmodulin (Anderson *et al.*, 1980).

Initially, therefore, I attempted to extract NAD kinase from pea seedlings by the method of Anderson and Cormier (1978) as described in chapter 2. The procedure involved homogenisation of pea shoot tissue in Tris and PVPP, followed by ammonium sulphate precipitation and ion exchange chromatography. NAD kinase activity was assayed by a modification of the method of Muto and Miyachi (1977).

The results of the extraction of NAD kinase from pea seedlings differed from those of Anderson and Cormier in two main respects. Firstly, NAD kinase was not detected in the void volume from the anion exchange column as expected, although it bound to the column and was eluted with 100-300mM NaCl as

shown in figure 3:3:1. Secondly, activity was not stimulated either by calcium, or by bovine heart (Sigma) or bovine brain calmodulin in the presence of calcium (figure 3:3:2).

Muto and Miyachi (1977) had observed that although the activator became separated from NAD kinase during ion exchange chromatography (under different conditions of extraction and chromatography), that the activator-dependent and activator-independent activities differed according to the salt concentration at which the fraction was eluted from the column. It was not clear whether this was due to the presence of different isozymes, to extract-generated forms, or to different degrees of binding of the activator to the enzyme. As it therefore seemed possible that NAD kinase had remained bound to its activator calmodulin, I observed the effects of heated aliquots of the NAD kinase preparation on calmodulin-dependent phosphodiesterase activity. No activation of phosphodiesterase could be detected as would be expected if calmodulin was present in the sample (figure 3:3:1). Furthermore, addition of phenothiazine inhibitors of calmodulin, or of calcium chelators to the NAD kinase assay did not reduce its activity (figure 3:3:2). It appeared, therefore, that calmodulin had not remained bound to the enzyme unless bound irreversibly in a calcium-independent manner.

The extraction procedure of Anderson and Cormier used Tris and PVPP for homogenisation. As described in chapter 3 section 2, this does not create conditions favourable to separation of calmodulin from calmodulin-binding proteins. As NAD kinase is present in plant cells in a far lower molar quantity than calmodulin (Muto, 1983), significant binding to membrane-bound calmodulin might occur. As calmodulin is found in at least a plasma membrane fraction of pea seedling (membrane fraction obtained from Dr. A. Hetherington), binding of NAD kinase to membrane-bound calmodulin might interfere with solubilisation at an early stage of extraction. Although this could potentially affect calmodulin-dependent NAD kinase, calmodulin-independent NAD kinase would not be affected if it is not capable of binding to calmodulin. Additionally, although some NAD kinase appears to be present in the cytoplasm, most is found in the chloroplasts

Figure 3:3:1. Extraction of NAD kinase and calmodulin by ion exchange chromatography by the method of Anderson and Cormier (1978)

NAD kinase was extracted from 50g of pea shoot tissue as described in chapter 2 section 2 H ii b. The tissue homogenate was taken to 50% saturation with ammonium sulphate, and the precipitate dissolved in and dialysed against 0.1mM EGTA, 25mM Tris pH 8, 0.1M NaCl. The sample was then passed through a 100ml DE-52 anion exchange column, and protein eluted with a linear gradient of 0-0.6M NaCl. Fractions of 5ml volume were collected, and assayed for NAD kinase activity as described in chapter 2 section 2 I ii, and for calmodulin as described in chapter 2 section 2 I i.

NAD kinase activity was assayed by incubating 100µl of each fraction in the presence of either 1mM $CaCl_2$ (——▲——), or 1mM $CaCl_2$ + 5µg bovine brain calmodulin (——●——).

To examine extracts for the presence of calmodulin, samples from each fraction were heated to 100°C for 2 minutes. The coagulated protein was removed by centrifugation, and 100µl aliquots of the sample were then used to activate bovine brain phosphodiesterase (——○——).

A_{280} values (——□——) and salt concentration of the fraction (——) are provided for comparison.
The results are expressed as pmoles NADP phosphorylated from NAD per 30 minute incubation.

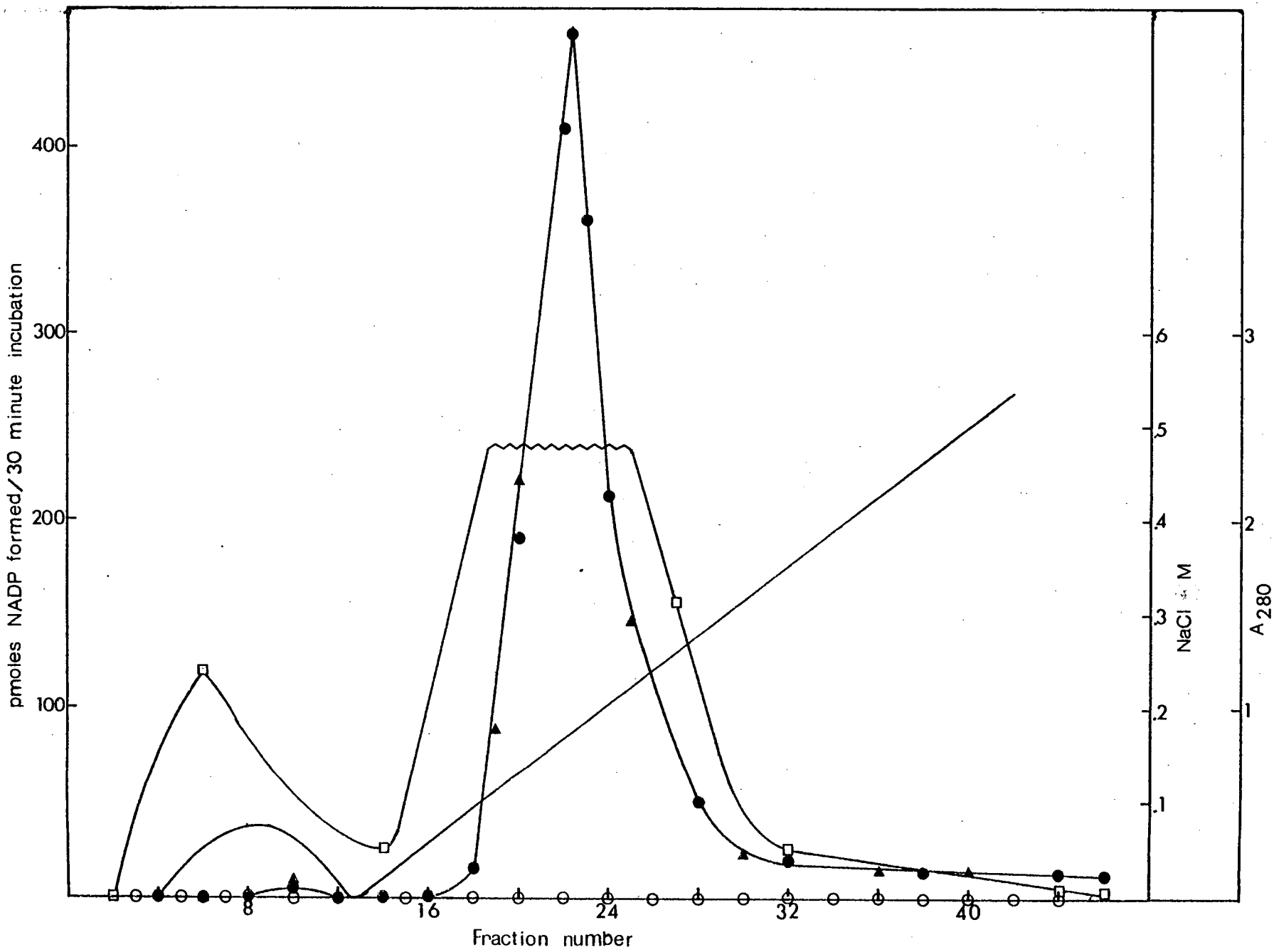

pmoles NADP formed/30 minute incubation
400
300
200
100
8
16
24
32
40
Fraction number
.6
.5
.4
.3
.2
.1
NaCl M
3
2
1
A280

Fig. 3:3:1.

Figure 3:3:2. The effect of calmodulin and calmodulin inhibitors on NAD kinase extracted by the procedure of Anderson and Cormier (1978)

NAD kinase was extracted, chromatographed on an anion exchange column, and eluted with a salt gradient as described in figure 3:3:1. 100µl aliquots from 5ml fractions from the ion exchange column were assayed for NAD kinase activity in the presence of 1mM $CaCl_2$ (——●——); 1mm $CaCl_2$ + 5µg of bovine brain or bovine heart calmodulin (——●——); 1mM $CaCl_2$ + 100µM trifluoperazine (——○——); or 2mM EGTA (——△——). The results are expressed as pmoles NADP formed from NAD per 30 minute incubation.

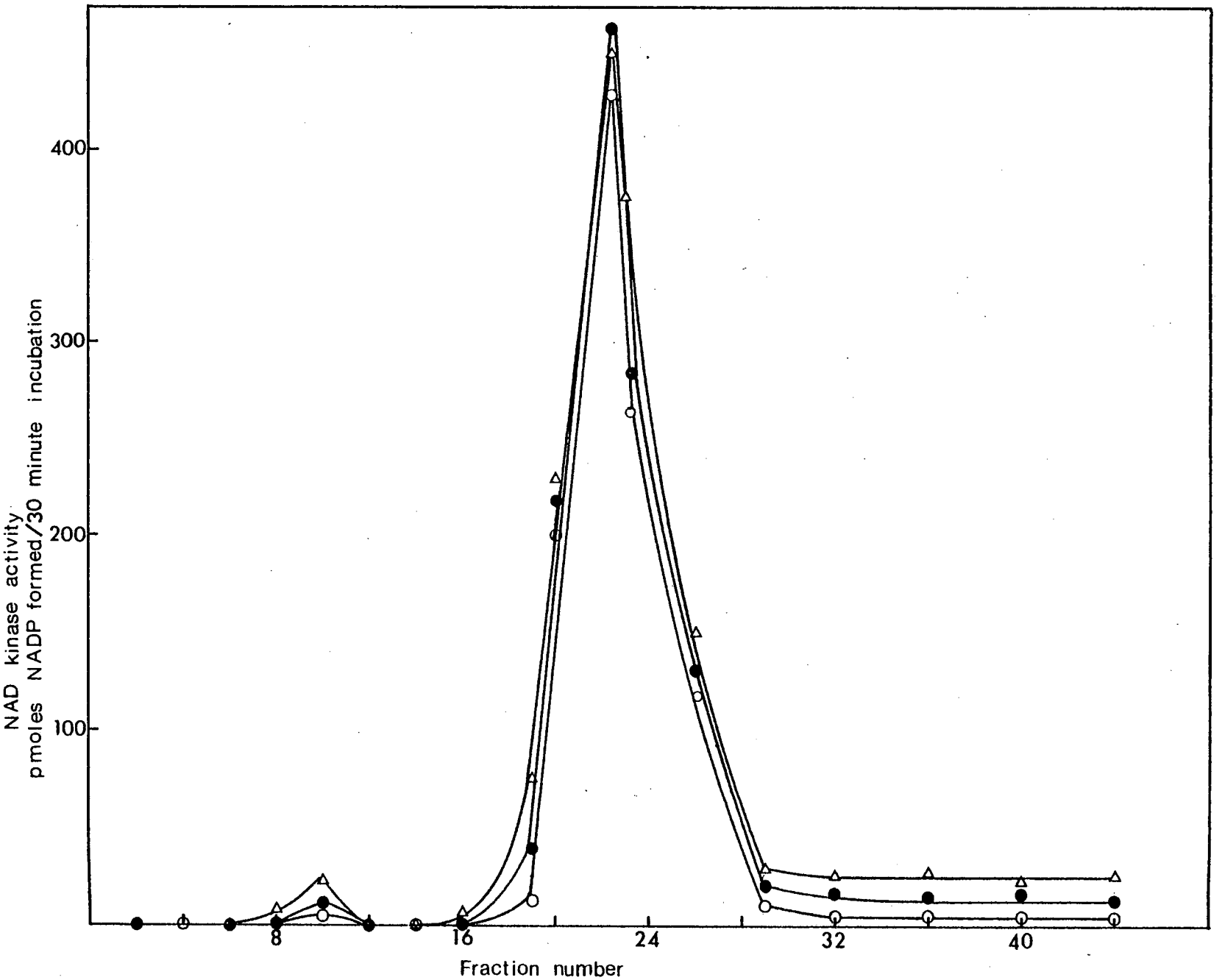

NAD kinase activity
pmoles NADP formed/30 minute incubation
400
300
200
100
8
16
24
32
40
Fraction number

Fig. 3:3:2.

of shoot tissue (Muto *et al.*, 1981; Jarrett *et al.*, 1982). It was therefore possible that calmodulin-dependent NAD kinase present in organelles or in membranes was removed by centrifugation early in the extraction procedure.

Anderson *et al.* (1980) had subsequently developed a method for improved extraction of NAD kinase employing high salt concentration and EGTA in the homogenising medium and I felt that this procedure might extract calmodulin-dependent NAD kinase from pea seedlings. I therefore attempted to extract NAD kinase by this method.

(ii) The procedure of Anderson *et al.* (1980)

Extraction of NAD kinase from pea shoot tissue was carried out by the method of Anderson *et al.* (1980) as described in chapter 2. This procedure involved homogenisation in a buffer containing EGTA and 1M KCl, ion exchange chromatography, ammonium sulphate precipitation, and passage of the redissolved pellet through a second ion exchange column.

A far greater yield of NAD kinase was obtained by this procedure, and both calmodulin-dependent and calmodulin-independent NAD kinase activities were detected as shown in figure 3:3:3. In crude homogenates of pea shoot tissue, calmodulin-independent NAD kinase activity comprised about 7% of total potential activity when fully activated with bovine brain calmodulin and calcium. These activities could be separated on the anion exchange column. Calmodulin-deficient NAD kinase that was completely dependent on added calmodulin and calcium for activity was eluted at 0.1M KCl. Activation was completely abolished by calcium chelators or by low concentrations of the calmodulin inhibitor, trifluoperazine. Half maximal inhibition occurred at 22μM trifluoperazine, and complete inhibition at 80μM (figure 3:2:5). All calmodulin-dependent activity loaded onto the column was eluted in this fraction. On the other hand, all calmodulin-independent activity loaded on to the column was eluted at a higher salt concentration of 0.4M KCl. Results are shown in figure 3:3:3 and table 3:3:1.

These results do not exactly correspond to those of

Figure 3:3:3. Extraction of NAD kinase by the method of Anderson *et al.* (1980), and separation of calmodulin-dependent and calmodulin-independent NAD kinase activities by ion exchange chromatography

NAD kinase was extracted from 100g pea shoot tissue as described in chapter 2 section 2 H ii a. The tissue was homogenised in buffer containing 1mM EGTA and 1M KCl, centrifuged at 12,000 x g, and the pellet discarded. The supernatant was passed through a DEAE Sephacel ion exchange column equilibrated at 0.5M KCl, and the eluate brought to 50% saturation with ammonium sulphate. The pellet was then dialysed against a buffer containing 0.1M KCl, and the 24,000G supernatant passed through a second anion exchange column and eluted with buffer containing 0.1M KCl as shown in figure 3:3:3. After most of the protein eluting at 0.1M KCl had been removed from the column (Fraction 32), KCl was added to the elution buffer to a final concentration of 0.4M to remove proteins binding to the column more strongly. 10ml fractions were collected, and 50µl aliquots removed for assay of NAD kinase activity and for estimation of calmodulin. NAD kinase assays were carried out in the presence of 1mM $CaCl_2$ (——▲——); 1mM $CaCl_2$ + 5µg bovine brain calmodulin (——●——); 1mM $CaCl_2$ + 5µg bovine brain calmodulin + 100µM trifluoperazine (——▲——); 2mM EGTA (——▲——); or 2mM EGTA + 5µg bovine brain calmodulin (——▲——). A_{280} values (——□——) are provided for comparison.

The results are expressed as pmoles of NADP phosphorylated from NAD per 30 minute incubation.

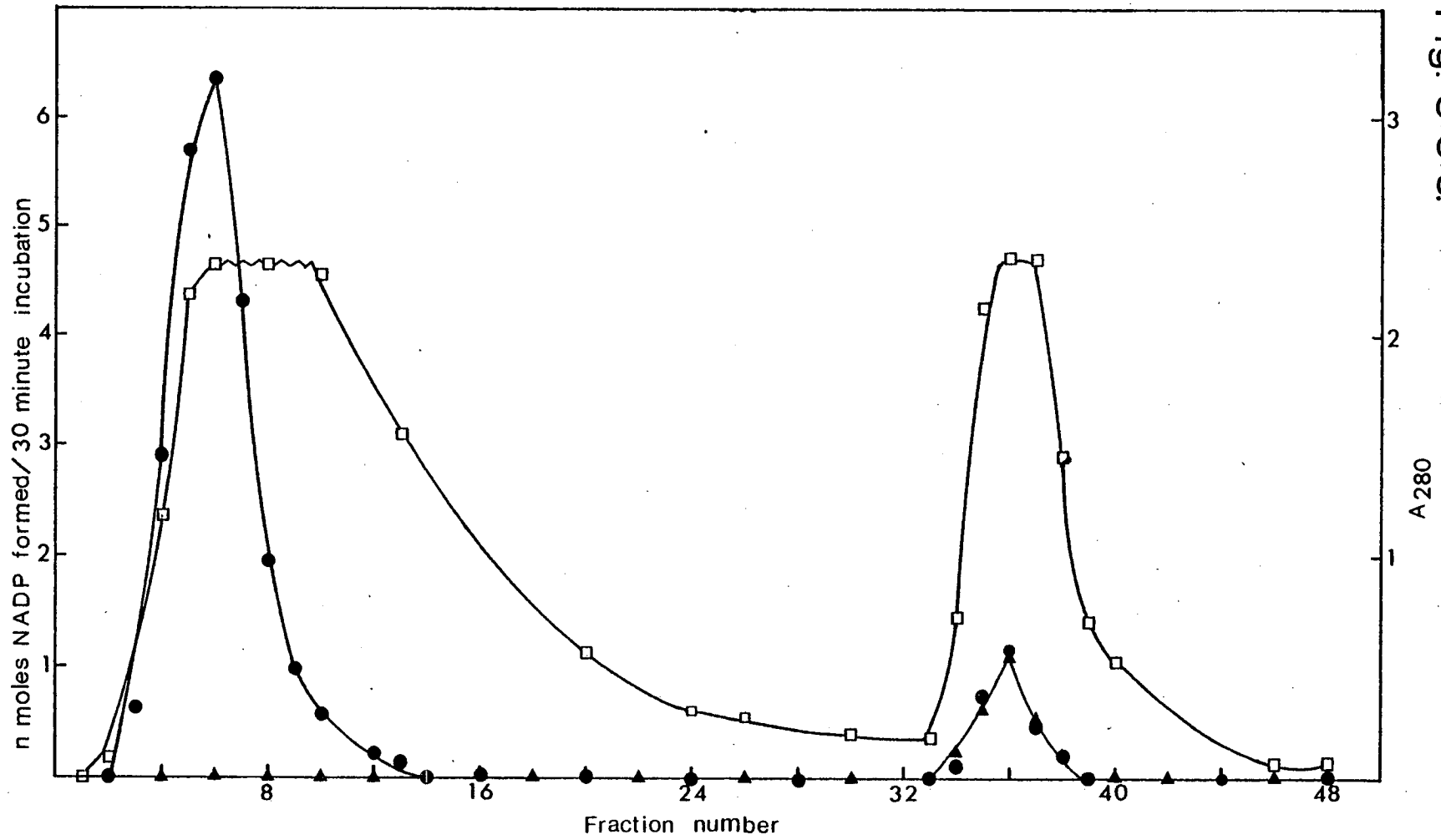

n moles NADP formed/ 30 minute incubation
A280
Fraction number
8
16
24
32
40
48
1
2
3
4
5
6

Fig. 3:3:3.

Table 3:3:1. Calmodulin-dependence of NAD kinase activity in pea shoot tissue

Treatment	NAD kinase activity (pmoles NADP/30 minute incubation) Crude homogenate 25µl	0.1M KCl eluate 50µl	0.4M KCl eluate 50µl	0.4M KCl eluate heated. 50µl
+ 1mM $CaCl_2$	2835	10	1160	0
+ 1mM $CaCl_2$ + 5µg bovine calmodulin	3880	6330	1260	0
+ 2mM EGTA + 5µg bovine calmodulin	280	0	1200	0
+ 1mM $CaCl_2$ + 5µg bovine calmodulin + 80µM trifluoperazine	70	0	884	0
+ 2mM EGTA	270	0	1150	0
Phosphodiesterase activation[1]	0	0	0	0
NAD kinase activation[2]	-	-	-	0

1. Corrected for background activity.
2. Assayed with 50µl of the 0.1M KCl eluate in the presence of 1mM $CaCl_2$.

NAD kinase was extracted by the method of Anderson *et al.* (1980) as described in figure 3:3:3, and aliquots from the ion exchange column were assayed for NAD kinase activity as described in chapter 2 section 2 I ii. Phosphodiesterase activity was assayed by the procedure of Cheung (1971). Samples of NAD kinase were incubated in the presence of calmodulin or under calmodulin-inhibiting conditions to estimate calmodulin-dependent activity.

Anderson *et al.* The NAD kinase activity that they obtained from a 0.1M KCl eluate was activated 7-30 fold by calmodulin, and they suggested that this was due to the degree of contamination by calmodulin in the sample. Unfortunately they did not appear to have tested the activity of this fraction in the presence of calcium chelators or calmodulin inhibitors, and it is therefore not certain whether this NAD kinase sample was completely or partially dependent on calmodulin.

I had noted that trifluoperazine consistently produced a slightly greater inhibition of NAD kinase in crude extracts than did EGTA. I therefore examined the possibility that calmodulin had remained attached in a calcium-independent manner to the calmodulin-independent NAD kinase eluted at 0.4M KCl. When I tested the ability of heat-treated samples of calmodulin-independent NAD kinase to activate calmodulin-dependent NAD kinase or phosphodiesterase, no activation could be detected. Neither could activity of the 0.4M KCl eluate be abolished by phenothiazine inhibitors or by EGTA (table 3:3:1). It is therefore unlikely that calmodulin is present in the sample of calmodulin-independent NAD kinase.

Calmodulin-independent activity does not appear to represent basal activity of a single enzyme without its activator, as the calmodulin-dependent and independent activities separate during ion exchange chromatography. Furthermore, there is only one known example of genuine calcium-independent binding of calmodulin to an enzyme, phosphorylase b kinase (Cohen *et al.*, 1980). In this case, calmodulin is present as a calcium-independent subunit of the enzyme. The enzyme has low activity in this form. Upon the calcium-dependent binding of a second molecule of calmodulin activity is greatly increased in a calcium-dependent manner. There is no evidence to suggest that this type of regulation also occurs with NAD kinase.

It therefore seems probable that there are two forms of NAD kinase, occurring either due to an artifact of extraction or to the presence of two different isozymes. As extraction of calmodulin-independent NAD kinase can occur in the presence of traces of calcium and absence of salt whereas the calmodulin-dependent form requires the presence of EGTA for its extraction,

it seems possible that the calmodulin-dependent form binds to calmodulin present in membranes or remains in the chloroplast and is removed by centrifugation during extraction in the presence of calcium, whereas the calmodulin-independent form does not bind to calmodulin either in the presence of calcium or EGTA. Thus both forms may be extracted by the method of Anderson *et al.* although only the calmodulin-independent form by the method of Anderson and Cormier. This is not dissimilar to the observation of Dieter and Marmé (1980c) that ATP-dependent Ca^{2+} uptake into a *Zucchini* microsomal fraction is only stimulated by calmodulin if the microsomes are prepared in the presence of EDTA, presumably as endogenous calmodulin has to be removed.

In subsequent experiments to further purify calmodulin-dependent NAD kinase, Jarrett *et al.* (1980) found that a sample of NAD kinase purified by calmodulin-affinity chromatography could be stimulated 100 fold by calmodulin and calcium. The enzyme NAD kinase was therefore claimed to be completely dependent on calmodulin and calcium for activity (Jarrett *et al.*, 1980; Charbonneau *et al.*, 1980). Although it was observed that calmodulin-independent activity was about 10% of total activity in the crude homogenate, becoming lower in subsequent stages of purification (Jarrett *et al.*, 1982b; Cormier *et al.*, 1982), it was suggested that the calmodulin-independent form was a minor or non-existent species. Unfortunately, although the calcium eluate from the calmodulin-affinity column was estimated in the presence of added calmodulin and calcium, neither the activity in the absence of calmodulin, nor the activity in the presence of EGTA or calmodulin inhibitors appear to have been estimated. This leaves open the possibility that the calcium eluate contained calmodulin-independent NAD kinase activity that did not bind to the affinity column. Fractions discarded during purification prior to affinity chromatography did not appear to have been examined for NAD kinase activity either, and it is therefore possible that calmodulin-independent activity would be found in these fractions.

Although Cormier *et al.* (1981) claim that they found in previous work (Anderson *et al.*, 1980; Anderson and Cormier,

1978) that NAD kinase in the presence of calmodulin was inactive in submicromolar levels of calcium, this does not, in fact, appear to be the case from their data. It would appear that only the calmodulin-dependent stimulation of NAD kinase is calcium-dependent (see Anderson and Cormier, 1978; Anderson *et al.*, 1980) as addition of EGTA to NAD kinase did not abolish calmodulin-independent activity which comprised about 20% of this extract. From their results, therefore, it appears that they obtained both calmodulin- and calcium-independent, and calmodulin- and calcium-dependent activities.

Most NAD kinase activity in shoot tissue is present in chloroplasts (Muto *et al.*, 1981; Jarrett *et al.*, 1982a) and is light-dependent, thought to be the result of a light-induced influx of calcium ions to the chloroplast allowing association of calmodulin with calmodulin-dependent NAD kinase (Muto, 1982, 1983; Jarrett *et al.*, 1982). All work reported on calmodulin-dependent NAD kinase has been carried out on shoot tissue. However, both NADP and NADP-dependent enzymes are known to be present in root tissue, and presumably NAD kinase activity occurs in the root although with different primary functions. I therefore felt that it would be of interest to examine NAD kinase activity in root tissue to investigate the potential involvement of calmodulin in the regulation of pyridine nucleotides and the processes dependent on these coenzymes.

NAD kinase was extracted from pea roots by the method of Anderson *et al.* (1980). Again, both calmodulin-dependent and calmodulin-independent activities were found; however, the ratio varied according to the developmental stage as shown in figure 4:2:10). In root extracts, calmodulin-independent activity was a much higher proportion of total potential activity, providing 16-50% of potential activity compared to about 7% in shoot extracts. As discussed in chapter 5 the requirements for NADP, its uses, and possibly also its intracellular distribution will vary according to developmental position and metabolic activity in the root apex. The function of NADP in shoot tissue, however, is almost entirely concerned with photosynthesis, while NAD kinase is present largely in chloroplasts. It therefore seemed possible that different NAD kinase activities might have a developmental function, and I therefore further

examined the possibility that there were two forms of NAD kinase in pea seedlings one being completely dependent on calcium and calmodulin for activity, the other completely independent of both.

I had already observed that the activities could be separated on an anion exchange column. I felt that further purification of calmodulin-dependent NAD kinase and separation of the two forms by calmodulin-affinity chromatography would permit closer examination of the enzymes.

(iii) Affinity chromatography

The NAD kinase extraction procedures using calmodulin affinity chromatography of Dieter and Marmé (1980b) and Jarrett *et al.* (1980) do not involve the use of calcium chelators during extraction. I felt therefore that these procedures might allow considerable loss of calmodulin-binding proteins during extraction as indicated above. I therefore homogenised pea seedling tissue in a buffer containing EGTA and a high salt concentration to permit extraction of NAD kinase from chloroplasts and to maintain conditions for its separation from calmodulin. After dialysis to remove KCl, the sample was applied directly to a calmodulin ultrogel affinity column. Prior removal of calmodulin was not attempted by fluphenazine affinity chromatography as it is known that some calmodulin-binding proteins may also bind to this column in a calcium-dependent manner.

Material binding non-specifically to the calmodulin affinity column was removed by washing with a buffer containing calcium and KCl until the A_{280} value was less than 0.005. Material binding to the column in a calcium-dependent manner was then eluted with EGTA. Details of the method are given in chapter 2, section 2 H ii d and 2 H iii.

Both the calcium and EGTA eluates were tested for NAD kinase activity, and dependence on calmodulin and calcium. The results showed that although most activity passed through in the calcium eluate, the proportion of calmodulin-dependent to calmodulin-independent activities in the calcium and EGTA eluates differed considerably as shown in table 3:3:2.

Table 3:3:2. Partial separation of calmodulin-dependent and calmodulin-independent activities by calmodulin affinity chromatography

Tissue	NAD kinase activity (pmoles NADP/30 minute incubation)			
	+ 1mM $CaCl_2$	+ 1mM $CaCl_2$ + 5µg bovine calmodulin	+ 2mM EGTA	+ 1mM $CaCl_2$ + 100µM TFP
Shoot				
Crude homogenate (25µl)	3250	3520	240	200
Calcium eluate (50µl)	4550	5380	420	365
Calcium eluate immediately prior to EGTA elution (50µl)	0	0	0	0
EGTA eluate (50µl)	60	400	0	20
Root				
Crude homogenate (25µl)	985	980	460	380
Calcium eluate (50µl)	895	855	765	760
Calcium eluate immediately prior to EGTA elution (50µl)	0	0	0	0
EGTA eluate (50µl)	0	0	0	0

NAD kinase from pea tissue was extracted by calmodulin-affinity chromatography as described in the text. Aliquots from different fractions were assayed for NAD kinase activity by a modification of the method of Muto and Miyachi (1977) as described in chapter 2 section 2 I ii.

In shoot tissue, 7% of total protein activity loaded on to the column was calmodulin-independent. However, all activity retained on the column in a calcium-dependent manner was entirely dependent on calcium and calmodulin. Most calmodulin-dependent activity passed through in the calcium eluate, presumably remaining bound to endogenous calmodulin which is present greatly in excess of NAD kinase (Muto, 1983). With root tissue, 60% of the total potential activity loaded on to the column was calmodulin-dependent. However, only 15% of NAD kinase activity in the calcium eluate was calmodulin-dependent indicating that calmodulin-dependent activity may have bound to the column in the presence of calcium. No activity was observed in the EGTA eluate, probably as the amount binding to the column was too low to be detected due both to the small amounts of tissue used for extraction and to the short half-life of NAD kinase activity; and to interference with the NAD kinase assay by other calmodulin-binding proteins in the EGTA eluate which would be expected to compete for calmodulin, and which may include calmodulin-binding inhibitory proteins.

These preliminary results indicate that the calmodulin-dependent NAD kinase activity appears to bind to a calmodulin affinity column in the presence of calcium, whereas the calmodulin-independent activity does not.

These results do not rule out the possibility that the calmodulin-independent form of NAD kinase is extract-generated or has calcium-independent binding to calmodulin that remains relatively unaffected by calmodulin inhibitors, particularly as it was noted that trifluoperazine inhibited crude extracts of NAD kinase to a greater extent than did EGTA. However, as the calmodulin-independent activity cannot be abolished by calmodulin inhibitors (figures 3:3:2 and 3:3:3) and will not, after ion exchange chromatography followed by heat treatment, activate calmodulin-dependent NAD kinase or phosphodiesterase (figure 3:3:1) it seems unlikely that activity is due to calcium-independent binding of calmodulin to NAD kinase. It was also noted that the total extracted calmodulin-independent NAD kinase activity is similar whether extraction is carried out by the method of Anderson and Cormier or Anderson *et al.* although

the extraction of the calmodulin-dependent form does depend on the extraction procedure (compare figures 3:3:1 and 3:3:3). This would not be expected if the calmodulin-independent form was extract-generated.

The calmodulin-independent activity also does not appear to represent basal activity in the absence of calmodulin, as the calmodulin-dependent and -independent activities may be separated by ion exchange and calmodulin affinity chromatography (figure 3:3:3 and table 3:3:2). Furthermore, the activities are present in different ratios in the root and shoot (table 3:3:2), while the ratio varies throughout the root depending on the distance from the apex (figure 4:2:10). Activators other than calmodulin have been found for a variety of other calmodulin-dependent enzymes (Scharff, 1981); however, Jarrett *et al.* (1982a) have not observed any other activators of NAD kinase such as lipids or proteins, including proteases. The calmodulin-independent activity is therefore unlikely to be due to an activator other than NAD kinase.

These preliminary results indicate that there may be two forms of NAD kinase. The activity of one of these is independent of calmodulin and calcium. This form may be readily obtained by gentle extraction methods. It elutes from an anion exchange column at neutral pH with a relatively high salt concentration, and does not bind to a calmodulin-affinity column. It contributes very little of the potential NAD kinase activity in shoot extracts, however, it appears to provide a much higher proportion of NAD kinase activity in root extracts. The other form requires harsher conditions for extraction, probably to release it from calmodulin attached to membranes and from mitochondria and plastids. This form is completely dependent on calmodulin and calcium for activity, and binds to a calmodulin affinity column. It elutes during anion exchange chromatography at a lower salt concentration than the calmodulin-independent form, and is presumably therefore a more basic protein. This form provides almost all the potential NAD kinase activity in shoot extracts, although far less in root extracts.

The possibility that the calmodulin-independent NAD kinase contains calmodulin bound in a calcium-independent manner could

be investigated further by purification of both forms of NAD kinase to permit amino acid sequencing or peptide mapping by limited proteolysis in SDS polyacrylamide gels. This would also help to distinguish between the possibilities that there are two isozymes of NAD kinase in pea seedlings or that one of the forms is generated by modification during extraction.

B. Estimation of the concentration of calmodulin in pea seedlings by NAD kinase activation

(i) Introduction

Calmodulin-deficient calmodulin-dependent NAD kinase was obtained by the method of Anderson *et al.* (1980) as described in section 3 A ii. As the calmodulin-independent form was removed by ion exchange chromatography, this preparation of NAD kinase was completely dependent on added calmodulin and calcium for activity as shown in figures 3:3:3 and 2:6 and table 3:3:1, while activation could be completely abolished by calmodulin inhibitors of calcium chelators (figure 3:3:3). I therefore examined the NAD kinase assay procedure of Muto and Miyachi (1977) using this extract to assess whether it would be a useful procedure for estimating calmodulin obtained from pea root tissue.

(ii) Characterisation of the NAD kinase assay system:

a) Optimisation of the assay system

The NAD kinase assay procedure of Muto and Miyachi (1977) involved incubation of NAD kinase with its substrate to produce NADP. The NADP formed in this stage was then assayed in a second stage after G-6Pdh catalysed conversion to $NADPH_2$. The $NADPH_2$ formed was assayed by reduction of PMS which in turn reduced 2,6-DCPIP. Reduction of 2,6-DCPIP was measured spectrophotometrically by a decrease in absorbance at 600nm.

The original assay system was found to be unable to detect quantities of NADP below millimolar levels. NAD kinase samples were therefore required to be fully activated by

relatively large amounts of calmodulin before activity could be detected. Furthermore, the enzymatic assay of NADP was found to be rapidly inhibited, resulting in a non-linear decrease in absorbance at 600nm. I therefore attempted to increase the sensitivity of the assay system and to reduce inhibition of stage II.

Several of the chemicals used in the NAD kinase buffer and in the first stage of the NAD kinase assay were found to inhibit both the NAD kinase incubation and the NADP assay. $MgCl_2$ is present in the assay at 10mM in stage I and 5mM in stage II. At these concentrations $MgCl_2$ was found to strongly inhibit stage II, inhibition increasing as $MgCl_2$ concentration was raised. It also inhibited stage I although to a lesser extent than stage II; although reducing Mg to below 8mM reduced activity of stage I. EDTA was found to release the inhibition by $MgCl_2$, and $MgCl_2$ was therefore kept at the original concentration in the presence of EDTA. Similarly, Tris, which is present at 100mM pH8 in stage I and 250mM pH8 in stage II, strongly inhibited both stages at these concentrations. Alteration of pH values between 6 and 8.5, and substitution of HEPES for Tris did not reduce the inhibition. Optimum concentrations of 40mM Tris pH8 in stage I and 20mM pH8 in stage II were finally used. EDTA was found to relieve inhibition by Tris considerably, as for $MgCl_2$.

KCl, which is used in extraction of NAD kinase was also found to inhibit the assay. However, at the concentration at which it is present in the assay of calmodulin, 10mM, inhibition was minimal. $MgCl_2$ and Tris are also present in the NAD kinase extraction buffer, but again at too low a concentration to inhibit activity. Neither EGTA nor trifluoperazine, which were used to inhibit calmodulin-dependent activation of NAD kinase, inhibited the stage II incubation at concentrations of up to 10mM EGTA and 400μM or higher trifluoperazine. NAD was found to have no significant effect on stage II incubation. However, one batch of G-6-Pdh (Sigma) was observed to have NAD-dependent activity, and the results obtained from experiments using this batch were discarded.

In addition to relieving inhibition by Tris and $MgCl_2$,

EDTA also released the inhibitory effect on stage II that resulted in a non-linear decrease in absorbance at 600nm. EDTA was therefore included in both stages of the assay.

An increase in G-6-P and G-6-Pdh were found necessary to increase sensitivity of the assay system to detect pmole levels of NADP.

The final assay system therefore contained a considerably reduced concentration of Tris in both stage I and stage II, while including EDTA in both stages. G-6-P and G-6-Pdh were increased in stage II. Details are provided in chapter 2. NAD kinase assayed by this final procedure displayed a linear decrease in absorbance at 600nm. It was also 10^6 times more sensitive to NADP than the original procedure, having a range of detection of 1 pmole - 6 nmoles NADP, allowing detection of as little as 50ng of bovine brain calmodulin.

The enzyme was found to be very unstable at both 4°C and -20°C, having a half-life of 20 hours. Calmodulin-stimulated activity was therefore negligible by the third day after homogenisation. Neither 0.5mM PMSF, a protease inhibitor, nor 50% glycerol, improved stability. A fresh batch of NAD kinase was therefore prepared for each experiment, and stage I incubation was carried out 27-30 hours after harvesting. The enzyme was recalibrated with bovine brain calmodulin for each experiment.

b) Calibration of the calmodulin dependent activation of NAD kinase

Bovine brain calmodulin obtained by the method of Caldwell and Haug (1981a) was used to calibrate calmodulin-dependent activation of NAD kinase. Under the conditions specified, no NAD kinase activity could be detected in the absence of calmodulin and calcium. Although the specific activity of each batch of NAD kinase varied to some extent, full activation of 50µl of NAD kinase was always achieved between 2-4µg (235-470nM) bovine brain calmodulin and half maximal activation between 0.3 and 0.6µg (35-71nM). Full activation of 0.005 units of partially purified NAD kinase was reached at 4µg bovine brain calmodulin, with phosphorylation of 5 nmoles NAD/30 minute incubation 30 hours after harvesting the tissue. Half maximum activation

of 0.005 units occurred at 600ng. Results are shown in figure 2:6.

The concentration of calmodulin required for half maximal activation of NAD kinase at saturating calcium concentration is therefore similar to that for a variety of calmodulin-dependent enzymes (Scharff, 1981) including NAD kinase in wheat leaf and pea seedlings (Muto, 1983; Cormier *et al.*, 1981).

As the specific activity of each NAD kinase extract and the amount of protein/µl varied slightly, and as the enzyme was unstable, calmodulin-dependent activation of NAD kinase was recalibrated with bovine brain calmodulin for each experiment. Pea calmodulin was estimated by comparing the degree of activation by a given amount of extract with the activation by a known amount of bovine brain calmodulin. Measurements were taken from the linear section of the curve.

As discussed in chapter 3 section 2, although enzyme activating abilities of plant and bovine calmodulins were originally thought to be identical (Van Eldik *et al.*, 1980c; Jarrett *et al.*, 1980; Anderson *et al.*, 1980; Charbonneau *et al.*, 1980), more recent evidence indicates that they may not be the same. Recent evidence indicates that plant calmodulin is 7-fold more effective in activating NAD kinase than bovine calmodulin (Cormier *et al.*, 1981, 1982). It has also been found (Charbonneau *et al.*, 1980; Cormier *et al.*, 1981) that low molecular weight fragments of 6-8,000 daltons, found in samples of calmodulin purified by affinity chromatography and which were probably fragments of calmodulin, could activate NAD kinase with a 30-fold lower ability than native plant calmodulin.

My own results using pea calmodulin purified by affinity chromatography indicate that there may be a difference in activating ability between bovine brain calmodulin and the sample that I obtained of pea calmodulin. However, they do not differentiate between the possibilities of the under-estimation of pea calmodulin by NAD kinase activation due to modification resulting in a reduction in specific activity, or of overestimation by NAD kinase activation due to a higher

specific activity of activation than bovine brain calmodulin and an overestimation of pea calmodulin by A_{276} values. To compare the specific activities of different calmodulins it will therefore be necessary to obtain purified unmodified pea calmodulin, and to compare the specific activity of this sample to that of bovine calmodulin. The estimation of the amount of calmodulin in pea tissue may then be recalibrated if necessary.

As it is therefore not certain whether the activating abilities of the bovine and pea calmodulins are identical, the estimation of pea calmodulin by NAD kinase activation using bovine brain calmodulin as a standard is at present only tentative. Estimation of the relative amounts of calmodulin in different sections of pea should not, however, be affected.

c) Interference with the NAD kinase assay

A variety of factors may interfere with calmodulin-dependent activation of NAD kinase, including calmodulin-binding proteins and calmodulin-like proteins. It was therefore preferable that both calmodulin and NAD kinase samples were purified as near to homogeneity as possible. Calmodulin was purified almost to homogeneity as determined by two-dimensional polyacrylamide gel electrophoresis (figure 3:2:7) as described in chapter 3 section 2. This included a heat treatment stage in the presence of EGTA to remove calmodulin-binding proteins. Calmodulin-dependent NAD kinase obtained by the method of Anderson *et al.* (1980), however, contained a large number of contaminating proteins as observed by polyacrylamide gel electrophoresis. As some of the components of this sample might interfere with the assay, in particular other calmodulin-binding, or calmodulin-like, proteins, I attempted to purify the enzyme further by calmodulin-affinity chromatography. However, although calmodulin-dependent NAD kinase was obtained in the EGTA eluate, the eluate contained several polypeptides as observed by two-dimensional polyacrylamide gel electrophoresis (shown in figure 3:2:10). As the enzyme was so unstable, further purification to remove these apparent calmodulin-binding proteins would have taken too long, and it was therefore not possible to use NAD kinase purified in this way for estimation of calmodulin. It was

therefore important to determine whether any factor in the sample of NAD kinase or calmodulin interfered with the assay.

NAD, NADH, NADP, $NADPH_2$ and ATP, all of which might interfere in the assay were removed in purification of NAD kinase during ammonium sulphate precipitation and dialysis. As the decrease in A_{600} was dependent on the presence of added NAD and ATP in stage I, certainly it appeared that NAD, NADP, $NADPH_2$ and ATP were not present in detectable amounts by this assay procedure.

To test for the presence of other promoters or inhibitors of NAD kinase in the calmodulin samples, each calmodulin sample was tested for NAD kinase activity, while activation of the calmodulin-dependent NAD kinase sample was tested for inhibition by EGTA and by trifluoperazine. Promoters of NAD kinase other than calmodulin did not appear to be present as no activation occurred in the presence of EGTA or low concentrations of trifluoperazine. None of the calmodulin samples displayed any NAD kinase activity. Neither was the activity of G-6-Pdh affected by the samples of calmodulin. However, a slight inhibition of NAD kinase activity occurred when increasing volumes of partially purified calmodulin from some zones of pea root were assayed. The inhibition was slight and there was no correlation between the concentration of calmodulin and degree of inhibition. It is therefore unlikely that inhibition by the calmodulin samples significantly reduced the estimation of calmodulin by NAD kinase assay. This could be further investigated by comparing activation of samples from different root zones homogenised separately and together.

Although it appeared unlikely that calmodulin-binding proteins were present in the calmodulin samples, it was possible that heat-stable calmodulin-binding proteins existed. This could be investigated by binding ^{125}I-calmodulin to polyacrylamide gels of the calmodulin sample, or by comparison of non-denaturing gels of the sample electrophoresed in the presence of calmodulin and calcium or EGTA. The NAD kinase extract could also be examined in this way.

C. NAD kinase activity in the root apex of pea seedlings

Preliminary estimates of the calmodulin-dependent and calmodulin-independent NAD kinase activities were obtained. NAD kinase was extracted from 100-200 1.25mm root sections using the homogenisation buffer of Anderson *et al.* (1980). The volume of the extract was too small to strain through cheesecloth as for shoot tissue, and the samples were therefore centrifuged at 25,000G for 30 minutes to remove PVPP and cell debris. Centrifugation at 25,000G in place of straining through cheesecloth and centrifugation at 12,000G did not alter activity of NAD kinase. Three-four hours after harvesting, all NAD kinase samples were incubated and assayed by the modified method of Muto and Miyachi. Calmodulin-independent activity was estimated by activity in the presence of calcium and trifluoperazine or in the presence of EGTA. Calmodulin-dependent activity was estimated by activity when fully saturated with bovine brain calmodulin less the calmodulin-independent activity.

Estimation of the NAD kinase activity in the crude homogenate is subject to greater interference than partially purified NAD kinase. Increasing the volume of NAD kinase to be assayed resulted in slight inhibition of some of the NAD kinase samples, largely as a result of a high concentration of KCl which inhibits the assay, and possibly also to the presence of NADH which is a potent inhibitor of NAD kinase (Muto, 1983). A large part of the inhibitory effect was removed if the samples were left for three hours before incubation, and entirely removed if dialysis was carried out prior to incubation, presumably at least partly as a result of the removal of NADH and KCl. The use of very small volumes of NAD kinase samples also minimised inhibition as increasing the volume of NAD kinase at less than 10µl produced linear increase in activity.

D. Summary

NAD kinase was extracted from pea seedlings by three methods. Two distinct types of NAD kinase activity were observed.

One form of NAD kinase activity was completely dependent on calmodulin and calcium for activity, and activity could be completely abolished by EGTA or by trifluoperazine. This form required high salt and EGTA during homogenisation for its extraction, probably to prevent it from binding to calmodulin in membranes or from remaining inside intact organelles. It may be separated from the other form of NAD kinase by anion exchange chromatography as it elutes at low salt concentration at neutral pH; and also by calmodulin-affinity chromatography as it binds to the affinity column in the presence of calcium. This form constitutes over 90% of the potential activity in crude homogenates of light-grown shoot extracts, slightly less in etiolated shoot extracts, but only 50-84% of total potential activity in extracts from the root apex.

The other form of NAD kinase is completely independent of calmodulin and calcium and is active in the presence of trifluoperazine or EGTA. It is extractable by gentle methods and is probably therefore present in the soluble fraction of plant cells. It is eluted at a higher salt concentration during anion exchange chromatography than the calmodulin-dependent form, presumably being more acidic. It does not bind to a calmodulin-affinity column. This form constitutes less than 10% of total potential NAD kinase activity in shoot extracts but 16-50% in root extracts, depending on developmental position.

The calmodulin-deficient, calmodulin-dependent NAD kinase was extracted from pea shoots by the method of Anderson *et al.* (1980) and the NAD kinase assay procedure of Muto and Miyachi (1977) was modified to become 10^6fold more sensitive to NADP, capable of detecting 50ng of bovine brain calmodulin. The calmodulin-dependent NAD kinase was then used to estimate the concentration of calmodulin in the pea root apex by comparing activation by highly purified root samples with activation by known amounts of bovine brain calmodulin.

NAD kinase activity and the ratio of calmodulin-dependent to calmodulin-independent activity were also examined in crude extracts from the root apex.

3:4 CHARACTERISATION OF AN *IN VITRO* TRANSLATION SYSTEM FROM WHEATGERM

Introduction

In vitro translation was used as a means of estimating the variety and concentration of mRNA species in different developmental stages of the root apex to provide an indication of whether control of protein synthesis during differentiation might be at the transcriptional level. Following incubation of pea root mRNA with (^{3}H) amino acids in a cell-free protein synthesising system, estimates of the relative amounts of translation products and their MWt and pI' values were obtained by fluorography of two-dimensional polyacrylamide gels. However, the products of *in vitro* translation need not reflect the complexity or relative abundance of input mRNA. Several simple precautions can be taken to reduce the problems of preferential translation, premature termination, and misreading of mRNA, and these are described in the following section.

A. Extraction of RNA from pea root tissue

A cell-free protein synthesising system derived from wheatgerm was used to translate mRNA obtained from different developmental stages in the root apex of pea seedlings. This system has been found to translate primarily cytoplasmic mRNA from plant tissue (Bottomley *et al.*, 1975); thus pea root mRNA which is not normally translated on 80S ribosomes may not be translated in this system, or may be translated only poorly.

RNA to be used for translation studies was obtained from pea seedlings by a slight modification of the method of Leaver and Ingle (1971) for the extraction of rRNA as described in chapter 2 section 2 J i. Modifications included homogenisation and phenol extraction in alkaline rather than neutral buffer to allow partitioning of mRNA into the aqueous phase (Brawerman *et al.*, 1972) by preventing the binding of 3'-terminal poly(A) segments of mRNA to denatured protein (Taylor, 1979). As total nucleic acids were extracted by this procedure, an additional

step which removed DNA, tRNA, 5SrRNA and carbohydrate by sodium acetate was also included as some of these components may inhibit translation (Palmiter, 1974; Kern, 1975; Kirby, 1965). The RNA samples obtained for translation therefore consisted largely of rRNA and mRNA. The final extraction procedure is described in chapter 2.

Loss of secondary structure of RNA has been found to improve translational efficiency and result in a more accurate reflection of the relative abundancies of mRNAs (Payvar and Schimke, 1979; Lodish, 1970). Prior to *in vitro* translation, therefore, aggregates of RNA formed by phenol extraction were dissociated and RNA was denatured by heating to 65°C for 10-15 minutes (Haines *et al.*, 1974; Longacre and Rutter, 1977; Kirby, 1965; Taylor, 1979).

The wheatgerm *in vitro* translation system was adapted from that of Marcu and Dudock (1974) as described in chapter 2 section 2 J. In this system, incorporation of (^{3}H) amino acids into TCA-precipitable material was found to be completely dependent on exogenous mRNA. The translation system was therefore characterised using pea root RNA to direct protein synthesis.

B. Conditions of *in vitro* translation

(i) Introduction

It is well established that the efficiency of translation of different mRNA species during *in vitro* translation may vary considerably depending on a variety of conditions, and that optimum conditions may also vary for different mRNAs. The relative abundance of translation products therefore does not necessarily reflect the relative abundance of input mRNA.

Alteration in non-specific components involved in protein synthesis may lead to specific as well as to non-specific changes in translation products through preferential translation (Lodish, 1971, 1974; Nuss and Koch, 1976; Lomedico and Saunders, 1977; Sonenshein and Brawerman, 1976), or misreading of the message, for example by premature termination (Tse and

Taylor, 1977) or termination readthrough (Atkins and Gesteland, 1975). The presence of, or competition for, specifically-required factors for individual mRNAs such as message-discriminating initiation factors (Lee-Huang and Ochoa, 1971; Wigle and Smith, 1973) will also affect the ratio of translation products.

When using *in vitro* translation as a means of estimating the complexity and abundance of input mRNAs it is therefore important to investigate conditions affecting translation of individual mRNAs as well as of total mRNA. This is particularly important with respect to components of the assay involved in initiation, as initiation appears to be a major control point for preferential translation (Lodish, 1971, 1974; Lodish and Jacobsen, 1972; Schreier and Staehelin, 1973; Lomedico and Saunders, 1977; Nuss and Koch, 1976) as a result of different abilities of mRNA species to form functional initiation complexes. As competitive conditions during initiation tend to result in preferential translation of mRNA species with high affinity for rate-limiting factors, these conditions should be avoided.

The concentration of RNA is particularly important in messenger competition, as saturating levels of mRNA may strongly or completely inhibit translation of specific mRNA species with low affinity for rate-limiting factors involved in initiation (Sonenshein and Brawerman, 1976, 1977; Herson *et al.*, 1979; McKeehan, 1974; Lomedico and Saunders, 1977). The cations Mg^{2+}, K^{+} and the polyamines such as spermidine and spermine, are also particularly important in determining not only total amino acid incorporation, but also the ratio of translation of different mRNAs (Wigle and Smith, 1973; Roberts and Paterson, 1973; Lodish, 1971; McKeehan, 1974; Hunter *et al.*, 1977) and length of translation product (Tse and Taylor, 1977; Hunter *et al.*, 1977; Benveniste *et al.*, 1976). As Mg^{2+} (Levin *et al.*, 1973; Wells and Beevers, 1975) and polyamines (Igarashi *et al.*, 1977, 1978) are involved in early stages of initiation of protein synthesis prior to or including mRNA binding to the small ribosomal subunit, the effect of the amount and ratio of translation products is probably due at least partly to the

effect on rate of initiation. However, as elongation rate also appears to be affected by these cations, and sub-optimal concentrations of these ions leads to premature termination (Tse and Taylor, 1977; Hunter *et al.*, 1977) it has been suggested (Hunter *et al.*, 1977) that the requirements for Mg^{2+}, K^{+} and polyamines for full-length translation products are a result of an increase in rate of chain elongation which will allow completion of synthesis of full-length products before mRNA is degraded.

(ii) Optimisation of the *in vitro* translation system

It is clearly necessary not only to optimise the cell-free protein synthesising system for total incorporation of amino acids into protein, but also to investigate translation products to determine whether any specific alterations with alteration in translation conditions has occurred. As different batches of wheatgerm vary in assay requirements (Taylor, 1979) it is necessary to characterise each batch of wheatgerm used. As the concentrations of RNA, Mg^{2+}, K^{+} and of a variety of factors contained in wheatgerm were particularly important to translation of specific mRNAs as well as to the general level of translation, the wheatgerm system was optimised for these using pea root RNA.

The cations Mg^{2+} and K^{+} were both found to affect translation. Low concentrations of either allowed only a poor rate of translation with a predominance of low MWt polypeptides. An increase in either ion increased incorporation of amino acids greatly, and increased the number of higher MWt polypeptides (figures 3:4:1 and 3:4:2). Magnesium was optimised in the presence of spermidine, as the presence of polyamines lowers the requirement for Mg^{2+}, while both are required for maximum incorporation and completion of full-length polypeptides (Igarashi *et al.*, 1975, 1978).

A requirement for Mg^{2+} and K^{+} for translation of high MWt polypeptides has been commonly observed, and appears to be due to completion of full-length polypeptides (Hunter *et al.*, 1977; Tse and Taylor, 1977). It was found that increasing Mg^{2+} between 2 and 2.5mM, and K^{+} between 100 and 135mM did not

Figure 3:4:1. The effect of K^+, Mg^{2+} and RNA concentration on *in vitro* translation products of pea root RNA

RNA from the apical 10mm of pea root was extracted as described in chapter 2 section 2 J i and used to direct protein synthesis in a wheatgerm S30 *in vitro* translation system. Translation conditions were as described in chapter 2 section 2 J iii with the following exceptions.

A. 80mM potassium acetate, 1.25mM magnesium acetate, 5.7µg RNA.

B. 80mM potassium acetate, 2.5mM magnesium acetate, 5.7µg RNA.

C. 130mM potassium acetate, 2.5mM magnesium acetate, 5.7µg RNA.

D. 120mM potassium acetate, 2.25mM magnesium acetate, 2.85µg RNA.

E. 120mM potassium acetate, 2.25mM magnesium acetate, 8.55µg RNA.

Fig. 3:4:1.

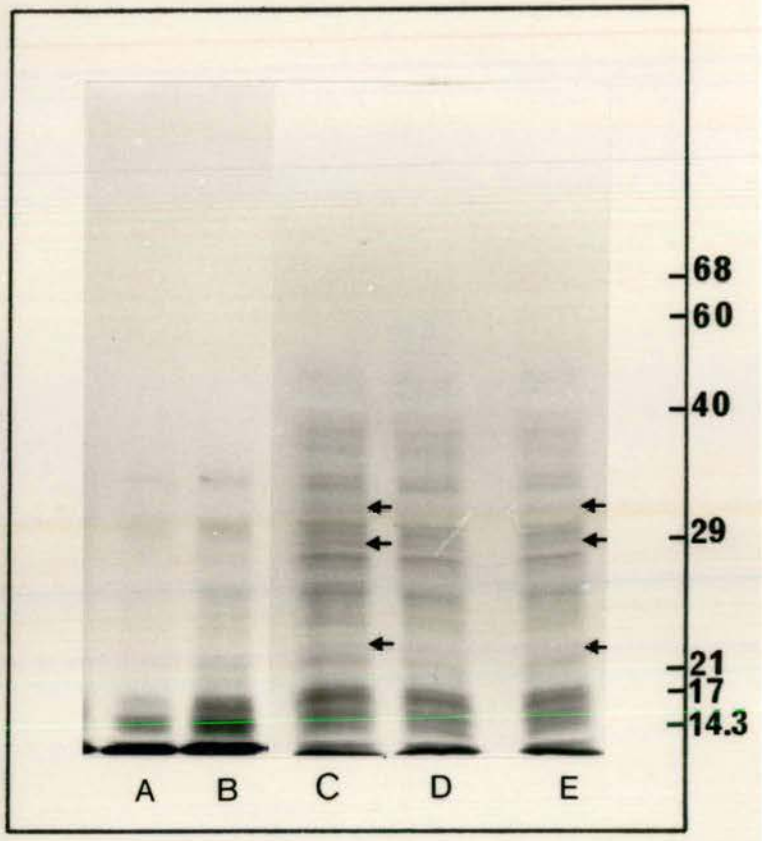


Fig. 3:4:2.

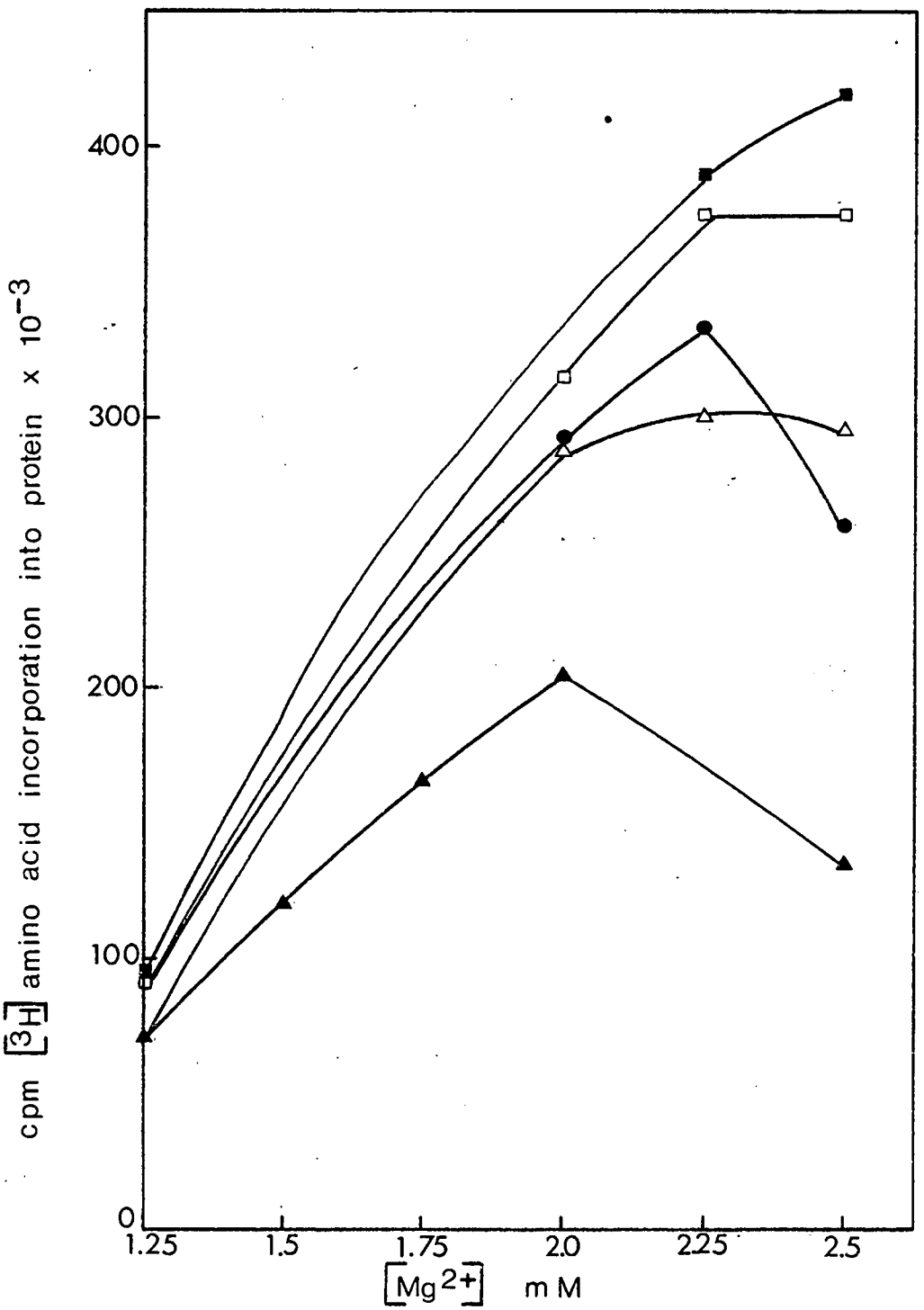

cpm [3H] amino acid incorporation into protein x 10-3
400
300
200
100
0
1.25
1.5
1.75
2.0
2.25
2.5
[Mg2+] m M

Figure 3:4:2. The effect of K^+ and Mg^{2+} concentration on incorporation of (^{3}H) amino acids into protein in an *in vitro* translation system directed by pea root RNA.

RNA from the apical 10mm of pea root was incubated in a wheatgerm S30 *in vitro* translation system as described in chapter 2 section 2 J, and the level of (^{3}H) incorporated into protein determined. Incubations were carried out for 90 minutes using 5.7µg RNA. Magnesium and potassium concentrations were varied. Potassium acetate concentrations in the final translation conditions were as follows:

—■— 130mM K^+

—□— 120mM K^+

—●— 110mM K^+

—△— 100mM K^+

—▲— 80mM K^+

alter relative amounts of different translation products, presumably as this concentration range was adequate for translation of full-length polypeptides. As has been generally found with the wheatgerm system (Marcu and Dudock, 1974; Roberts and Paterson, 1973), increasing Mg^{2+} and K^{+} above these levels led to a reduction in amino acid incorporation into protein. Optimum concentrations of 130mM K^{+} and 2.25mM Mg^{2+} were used in the final conditions for incorporation.

RNA concentration had a pronounced effect on amino acid incorporation into TCA-precipitable material (Figure 3:4:3). Incorporation increased with increasing RNA up to 6-10 µg RNA depending on the batch of wheatgerm. Incorporation declined thereafter, being almost totally inhibited by 30 µg RNA. A general decline in amino acid incorporation after peak incorporation with increasing RNA has been noted by others (Longacre and Rutter, 1977; Sonenshein and Brawerman, 1976) in addition to the decline in specific translation products as mentioned above.

When translation products obtained from incubations using 3-10µg RNA were observed by one-dimensional polyacrylamide gel electrophoresis, it was noted that two polypeptide bands, one at 22,000 and one at 29,000 were reduced relative to other translation products at the higher RNA concentrations where total amino acid incorporation was beginning to decline (Figure 3:4:1). One polypeptide at 32,000 daltons appeared on the other hand to be more prominent. This result could be interpreted in accordance with the model proposed by Lodish (1974) in which competition for rate-limiting factors involved in initiation will lead to preferential inhibition of translation of those mRNAs with low affinities for these factors. I did not examine the polypeptide pattern obtained from using more inhibitory concentrations of RNA. However, I investigated translation products further by two-dimensional polyacrylamide gel electrophoresis using another batch of wheatgerm in which incorporation was not limited by 10µg RNA (figure 3:4:3). In this case, no relative differences were observed in the translation products over a range of 3-10µg RNA. Thus preferential translation was not detected by two-dimensional gel electrophoresis over a non-limiting RNA

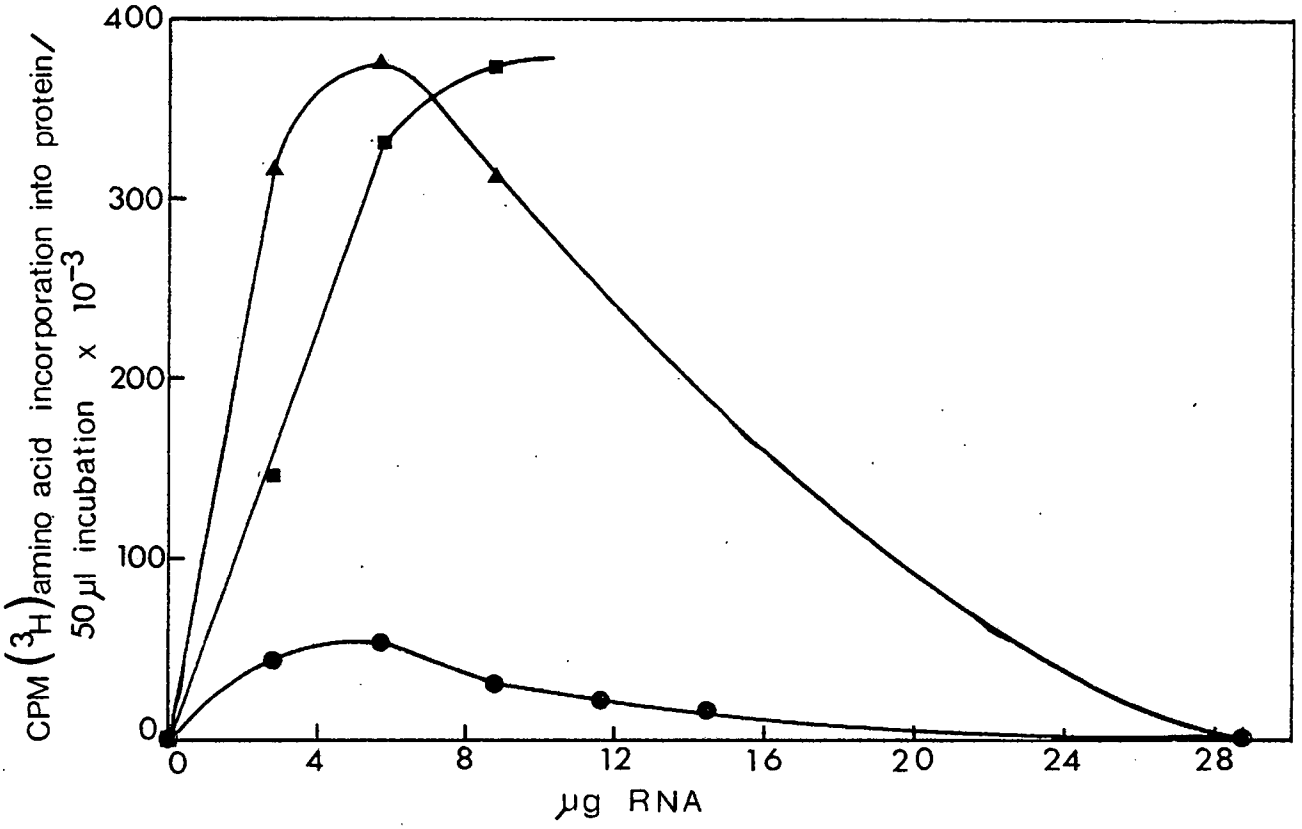
Figure 3:4:3. The effect of RNA concentration on (^{3}H) amino acid incorporation into protein in the wheatgerm *in vitro* translation system under different ionic conditions

RNA obtained from the apical 10mm of pea root was incubated for 90 minutes in the wheatgerm translation system as described in chapter 2 section 2 J.

RNA was used to direct protein synthesis in wheatgerm batch 1 in final concentrations of 80mM potassium acetate and 1.25mM magnesium acetate (——●——), or 120mM potassium acetate and 2.25mM magnesium acetate (——▲——).

RNA was also used to direct protein synthesis in wheatgerm batch 2 in the presence of 130mM potassium acetate and 2.5mM magnesium acetate (——■——).

Fig. 3:4:3.


CPM (^{3}H) amino acid incorporation into protein/ 50 µl incubation x 10^{-3}
400
300
200
100
0
0
4
8
12
16
20
24
28
µg RNA

concentration range. The latter batch of wheatgerm was therefore used for *in vitro* translation of pea root RNA, using a final concentration of 7µg RNA/50µl incubation.

The results do not, of course, rule out the possibility of complete or constant partial suppression of specific mRNA species, due for example to the absence of specific factors or to non-recognition of the initiator region of certain mRNAs. This possibility is indicated by the frequent observation that different *in vitro* translation systems produce different patterns of translation products (Cereghini *et al.*, 1979; Longacre and Rutter, 1977).

As wheatgerm contains initiation factors (Marcus *et al.*, 1974a) amongst a range of other factors potentially influencing translation specifically as well as non-specifically, the system was also optimised for wheatgerm. However, no relative differences in translation products were detected on polyacrylamide gels of translation products obtained using different amounts of wheatgerm. An optimum concentration of 10µl wheatgerm/50µl incubation was used in the final conditions for incorporation.

The time course for amino acid incorporation into TCA-precipitable material varied slightly with the conditions employed; however, after an initial lag period, incorporation was more or less linear up to 60 minutes, and was essentially completed by 90 minutes.

C. Identification of translation products

(i) Introduction

Translation products are frequently characterised by MWt and peptide mapping in SDS polyacrylamide gels, and by immunoreactivity. However, it has been observed that *in vitro* translation products may not resemble *'in vivo'* polypeptides using a variety of criteria including those mentioned above. This may be due to a variety of factors including production of precursor molecules (Cashmore *et al.*, 1978; Chua and

Schmidt, 1978), which may require some form of post- or co-translational modification such as phosphorylation, glycosylation or removal of a signal sequence (Campbell and Blobel, 1976) before they resemble their native counterpart. Alternatively, the message may not be read correctly, precursor RNA (Abelson, 1979) may be translated without complete processing, or nuclease activity may result in incomplete (Hunter *et al.*, 1977), or faulty translation. In view of the range of problems involved in identification of translation products, care has to be taken when comparing translation products with native polypeptides.

As translation products need not reflect either qualitatively or quantitatively the input mRNA, or resemble their *'in vivo'* counterparts, it was thought more useful to observe the translation of a specific identifiable polypeptide during *in vitro* translation of pea root mRNA. I therefore attempted to purify and identify calmodulin from the *in vitro* translation products, and to estimate its concentration.

(ii) Identification of calmodulin in the *in vitro* translation products

Calmodulin has been translated *in vitro* from spinach mRNA, purified from the translation products by phenothiazine affinity chromatography, and characterised and compared to native calmodulin (Van Eldik *et al.*, 1980a). With the exception of the absence of N'-trimethyllysine it appears to resemble calmodulin in all respects examined including mobility on polyacrylamide gels, calcium-dependent mobility shift during electrophoresis, and calcium-dependent binding to phenothiazines. It does not appear to be translated in precursor form.

I therefore attempted to isolate calmodulin from *in vitro* translation products by phenothiazine affinity chromatography. *In vitro* translation products equivalent to 3,000,000 cpm of TCA-precipitable material were loaded onto a phenothiazine-affi gel column in a calcium-containing buffer as described in chapter 2 section H i c II. After washing the column with the calcium buffer containing 0.3M KCl until radioactivity in the eluate had reached background level, material binding in a calcium-dependent manner to the column was eluted with EGTA

replacing $CaCl_2$ in the buffer. Aliquots were taken from the calcium and EGTA eluates and examined for TCA-precipitable radioactivity and for activation of calmodulin-dependent NAD kinase. Samples were also observed by one- and two-dimensional polyacrylamide gel electrophoresis.

An increase in radioactivity was observed in the EGTA eluate from the column, totalling 14,000 cpm after allowing for background radiation. This represents 0.5% of TCA-precipitable counts, about the estimated concentration of extractable calmodulin as a percentage of total protein in pea root tissue. However, although a substantial amount of calmodulin from wheatgerm itself bound to the column as detected by NAD kinase assay (1-2µg calmodulin/µl wheatgerm extract using bovine brain calmodulin as a standard) thus indicating that calmodulin bound to the column under these conditions, radioactively labelled calmodulin could not be detected by fluorography of one- or two-dimensional polyacrylamide gels of the EGTA eluate from the affinity column. The high estimate of the concentration of calmodulin in wheatgerm obtained by NAD kinase activation using bovine brain calmodulin as a standard indicates that calmodulin estimated in this way may be overestimated as a result of a higher affinity of plant calmodulin than bovine calmodulin for NAD kinase activation. This is supported by the observation that wheatgerm calmodulin could not be observed by staining gels of the EGTA eluate with Coomassie blue, with loading of 10µg of calmodulin according to the estimate by NAD kinase activation. Alternatively, it is possible that modification of calmodulin during purification occurred, as described by Charbonneau *et al.* (1980), to produce low MWt fragments of low specific activity which would not be observed by polyacrylamide gel electrophoresis. This is supported by the observation that neither the wheatgerm calmodulin nor the TCA-precipitable radioactive material eluted with EGTA from the phenothiazine column were observed on polyacrylamide gels or on fluorographs respectively.

As purified calmodulin was not detected on polyacrylamide gels after purification by affinity chromatography, calmodulin was not isolated from *in vitro* translation products for

quantitation. However, as Van Eldik *et al.* (1980a) had noted that translated spinach calmodulin comigrated with native calmodulin during electrophoresis, and underwent the characteristic calcium-dependent electrophoretic mobility shift, *in vitro* translation products were electrophoresed on two-dimensional polyacrylamide gels in the presence of either calcium or EGTA, and polypeptides examined for a calcium-dependent mobility shift during electrophoresis as shown in figure 4:2:5c and d.

D. Summary

A wheatgerm *in vitro* translation system directed by RNA from pea root tissue was optimised for incorporation of (^{3}H) amino acids into TCA-precipitable material. The system was also examined for any relative changes in translation products occurring as a result of changes in conditions of translation. Mg^{2+} and K^+ were found to affect translation particularly of high MWt proteins, and the system was optimised for these cations. RNA was present at non-limiting concentrations.

Isolation of calmodulin from *in vitro* translation products was attempted by phenothiazine affinity chromatography to identify and quantitate calmodulin in translation products. However, although radioactive material was eluted with EGTA from the column, and an NAD kinase activator from the wheatgerm incubation mix bound to the column in a calcium-dependent manner, calmodulin could not be observed on polyacrylamide gels. Calmodulin was therefore tentatively identified in translation products on two-dimensional polyacrylamide gels by a calcium-dependent electrophoretic mobility shift, and estimates made of its relative concentration in translation products.

CHAPTER 4

DEVELOPMENTAL FEATURES OF CELL DIFFERENTIATION AND MATURATION IN THE ROOT APEX OF *PISUM SATIVUM*

INTRODUCTION

The model of differentiation at the root apex developed by Brown (1963,1964) describes a progressive series of metabolic states which permit qualitatively different functions. Cells in the apical meristem are regarded as being in a dividing state, incapable of expansion and differentiation as a result of a particular enzyme complex. As a result of sequential regulation of transcription, cells develop an enzyme complement which permits expansion and differentiation, but which will not sustain division. Through an unequal cell division at the base of the meristem different protein and mRNA complements are partitioned in different cells, and with further growth these differences become apparent and constitute the process of differentiation. The same enzyme progressions are regarded as occurring in all cells at the same transverse level, but to different extents or at different rates. Through a progressive synthesis of regulators of mRNA transcription the protein complement continues to change, and eventually develops into an enzymic state in which growth cannot occur.

Many of the aspects of this model involve questionable assumptions as indicated in chapter 1. I therefore examined several anatomical and biochemical aspects of the model in order to assess whether such assumptions were justified and to further investigate the control of gene expression during differentiation in the root apex.

1. THE ORGANISATION OF THE APEX

A. Introduction

In order to be able to correlate molecular differentiation with cytological differentiation, and to examine the validity of regarding cells as in qualitatively different states at the anatomical level, the organisation of the apex was examined at the structural and ultrastructural levels. Characteristics of division, expansion and differentiation in individual tissues during development were examined, and the distances from the apex

at which differentiation and maturation could be recognised cytologically were assessed. The regions immediately adjacent to and distal to the quiescent centre were particularly closely studied in order to investigate characteristics of division and expansion from the apex of the root proper, and to investigate whether characteristics other than those resulting from cell division varied between apparently different groups of meristematic initials.

B. Structural development of tissues during differentiation

It has frequently been noted that age, length (Brown and Broadbent, 1950; Popham, 1955) and speed of growth of the root (Peterson, 1967; Heimsch, 1951) alter the size and shape of the apex, and the distance from the apex at which tissues differentiate and mature; slower growing roots displaying differentiation and maturation closer to the apex although the relative positions of tissue differentiation remain the same. Care was therefore taken to standardise growth conditions, and to select roots of approximately the same length from peas germinated for 65 hours. Roots were then fixed, and sections were obtained as described in chapter 2, section 2:C and D. Results are shown in figures 4:1:1-19 and tables 4:1:1-2.

The apical 20mm of pea root constitutes the main region of growth and differentiation, and areas within this region were therefore selected for study. All tissues present in roots of peas germinated for 65 hours appear to be initiated within the apical millimetre, while maturation of most cell types is virtually complete by 20mm (Table 4:1:1). It can be seen from figure 4:1:1 that differentiation is acropetal and continuous; differentiation constantly being initiated towards the apex as new cells form in the apex and 'grow away' from the older part of the root. Maturation, however, is basipetal; cells maturing as they progress with time from the apex. Growth, which consists of increase in both cell number and volume, continues throughout the entire 20mm (Figure 4:1:2, 4:1:3, 4:1:11) although by 20mm, cell division has almost entirely ceased and cell expansion is very slow. Cell divisions are then re-initiated 20-30mm from

Figure 4:1:1. The root apex of *Pisum sativum*

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 C ii. Median longitudinal sections of the apical 2mm were cut, and stained with toluidine blue for observation under the light microscope.

The apical region can be seen to be composed of the root proper surrounded at the distal end by the root cap (RC). The apical meristem lies at the boundary of the root cap and the root proper, the meristematic cells of the calyptrogen (CA) providing cells of the root cap; and meristematic cells of the procortex (PCX) and procambium (PCM) providing cells of the cortex and stele respectively.

Root cap cells may be readily distinguished at the boundary of the root cap and root proper by their large size, irregular walls, lightly staining cytoplasm, low vacuome content, and large starch grains. Procambial cells may be distinguished as elongated cells with small cross-sectional area containing a densely staining cytoplasm and a small number of small vacuoles. Procortical cells on the other hand are less elongated than procambial cells, but have a larger cross-sectional area, a large number of relatively large vacuoles, and a relatively weakly staining cytoplasm.

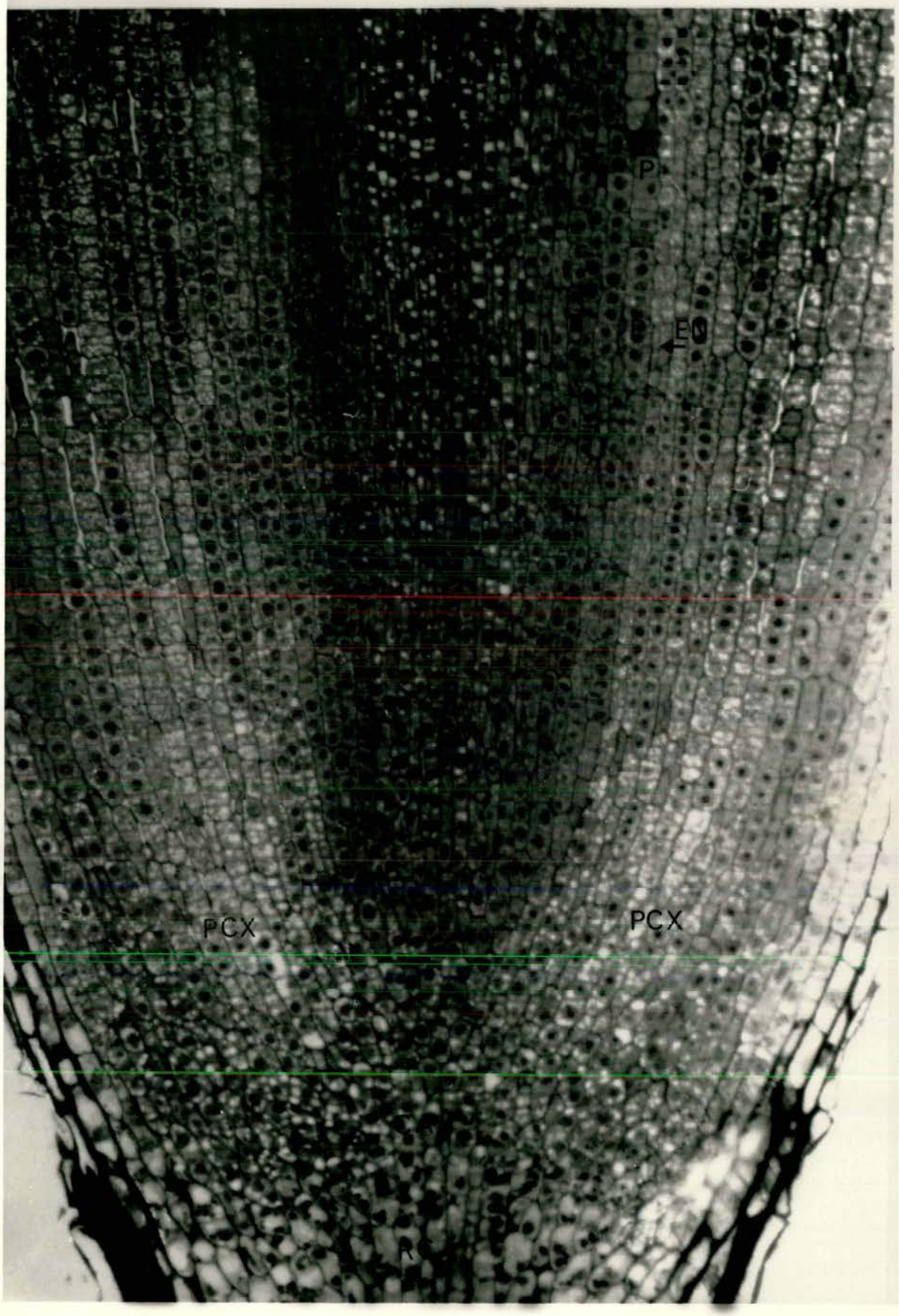
Several tissues emerge rapidly within the root proper. Pericycle cells (P) develop at the outer boundary of the procambial cylinder, distinguished from procambial cells by their weakly staining cytoplasm and low vacuome content, larger cross-sectional area, and shorter length. Adjacent endodermal cells (EN) lying on the internal boundary of the cortex are by contrast elongated and have a very narrow radial diameter and a high radial/tangential ratio. Several tissues develop from the procambial cylinder itself, including metaxylem which develops in the centre of the cylinder. These cells cease division, enlarge, and vacuolate earlier than adjacent phloem and protoxylem cells. Phloem and protoxylem may be readily distinguished in transverse section near the quiescent centre but are not readily distinguishable in longitudinal sections. Sieve elements (SE) may be distinguished from adjacent phloem at an early stage of differentiation by their weakly staining cytoplasm.

a) Pea root apex. Magnification x 158

b) Pea root apex. Magnification x 88

c) Promeristem and surrounding tissues. Magnification x 457.

Fig. 4:1:1. a



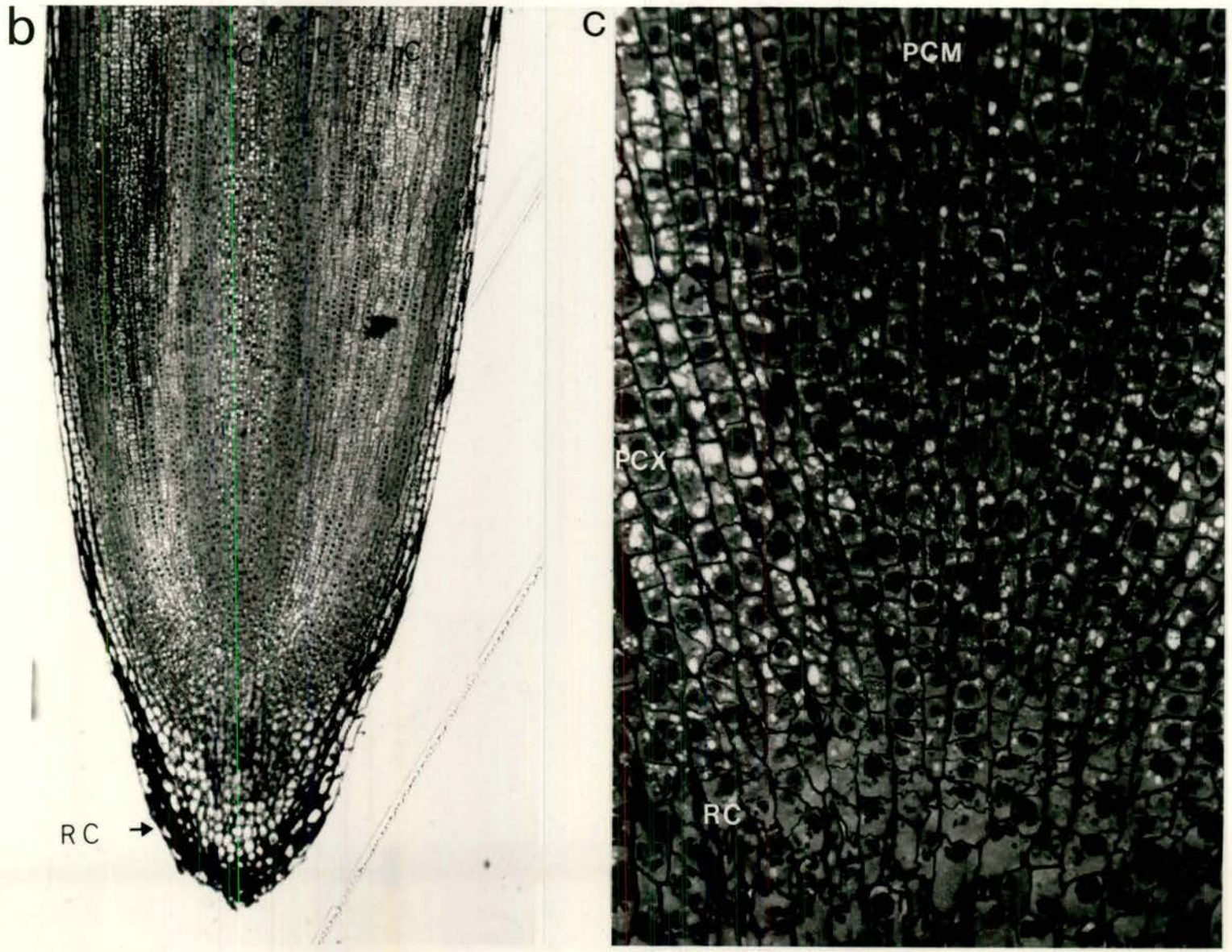

b
PCM
RC
c
PCM
PCX
RC

Table 4:1:1. The order and level of tissue differentiation in the pea root apex

Peas were germinated for 65 hours, and the roots were fixed, sectioned and stained for observation by the light and electron microscopes as described in chapter 2 section 2 C ii and D. The level of differentiation of a variety of tissues were then assessed using both anatomical and ultrastructural characteristics as discussed in chapter 4 section 1 D i.

	Distance from the root apex (μ)		Distance from the top of the columella (μ)	
Tissue	Range	Average	Range	Average
Root cap	0	0	-350 - -550	-425
Promeristem	350-550	425	0	0
Procortex	350-550	425	0	0
Procambium	380-600	470	30-50	45
Metaxylem	440-700	545	90-200	120
Endodermis	410-700	550	60-150	125
Pericycle	450-700	550	100-150	125
Phloem	420-850	625	70-300	200
Protoxylem	515-830	625	165-280	200
Protophloem[1]	600-1000	725	250-500	300
Protophloem[2]	700-1150	885	350-600	460
Protoxylem[3]	10,000-15,000	13,000	9,500-14,500	12,500
Protoxylem[4]	15,000-23,000	19,000	14,500-22,500	18,500

1. Protophloem sieve element differentiation
2. Protophloem sieve element maturation
3. Protoxylem with secondary wall thickenings
4. Protoxylem with lignified secondary walls

Figure 4:1:2. Change in cell number in individual cell layers during differentiation in the root apex

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C i or ii. Transverse sections were cut, and cell numbers in individual concentric layers of the root cap (——○——), endodermis (——▲——), pericycle (——△——), inner cortex (——■——) and mid cortex (——□——) were counted. The values refer to cell numbers in single cell layers in transverse section rather than to the entire tissue, and thus reflect longitudinal cell divisions.

Fig. 4:1:2.

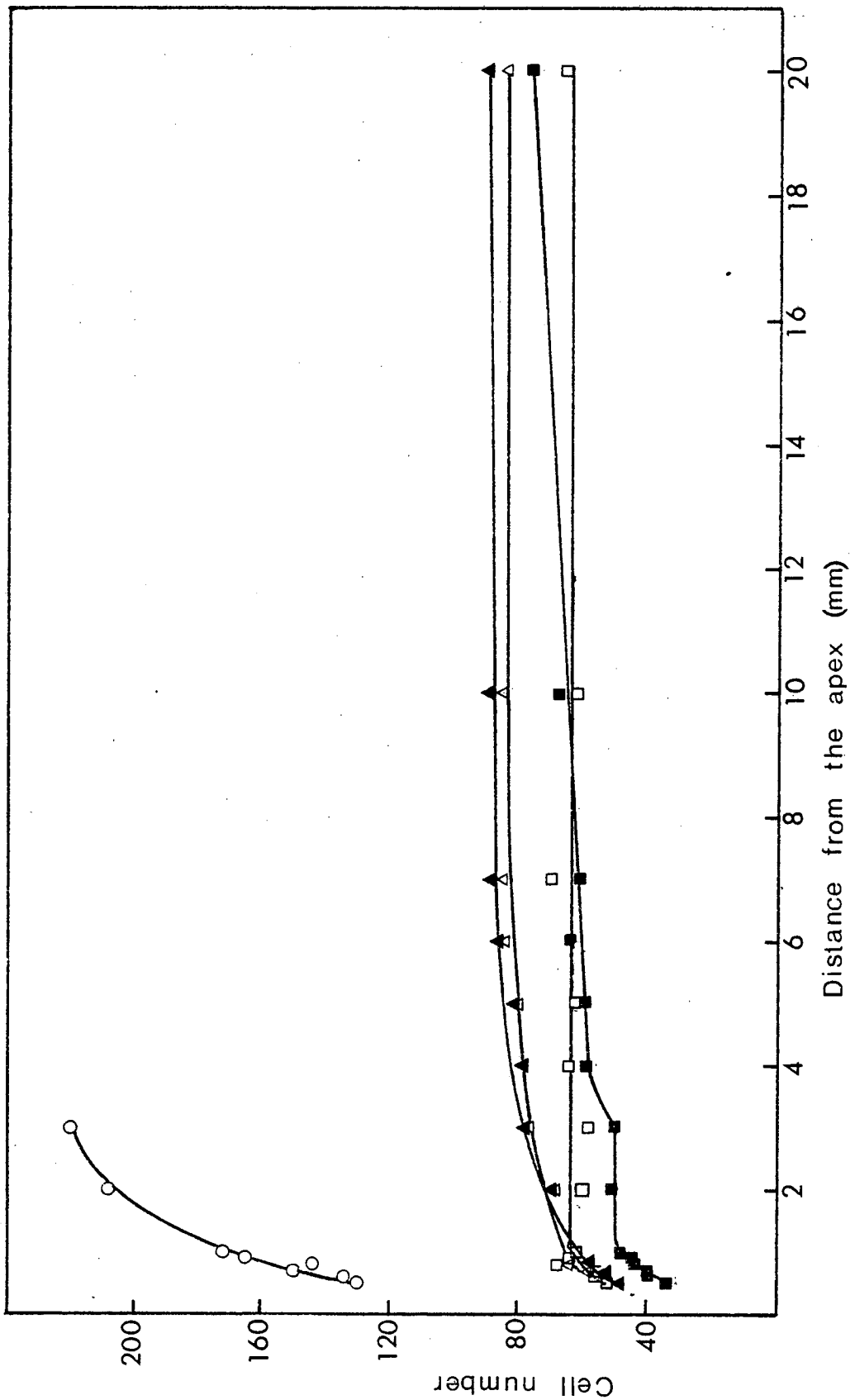

Cell number
40
80
120
160
200
Distance from the apex (mm)
2
4
6
8
10
12
14
16
18
20

Figure 4:1:3. Changes in cross-sectional area of tissues during differentiation in the pea root apex

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were cut at 100μ intervals within the apical millimetre, and at one millimetre intervals thereafter. The diameters of inner and outer tissue boundaries were measured, and the cross-sectional area then estimated under the assumption that the tissue boundaries were circular. Tissues estimated were the root cap (—○—), epidermis (—×—), cortex (—■—), endodermis (—▲—), pericycle (—△—), stele (—□—), and total root area (—●—).

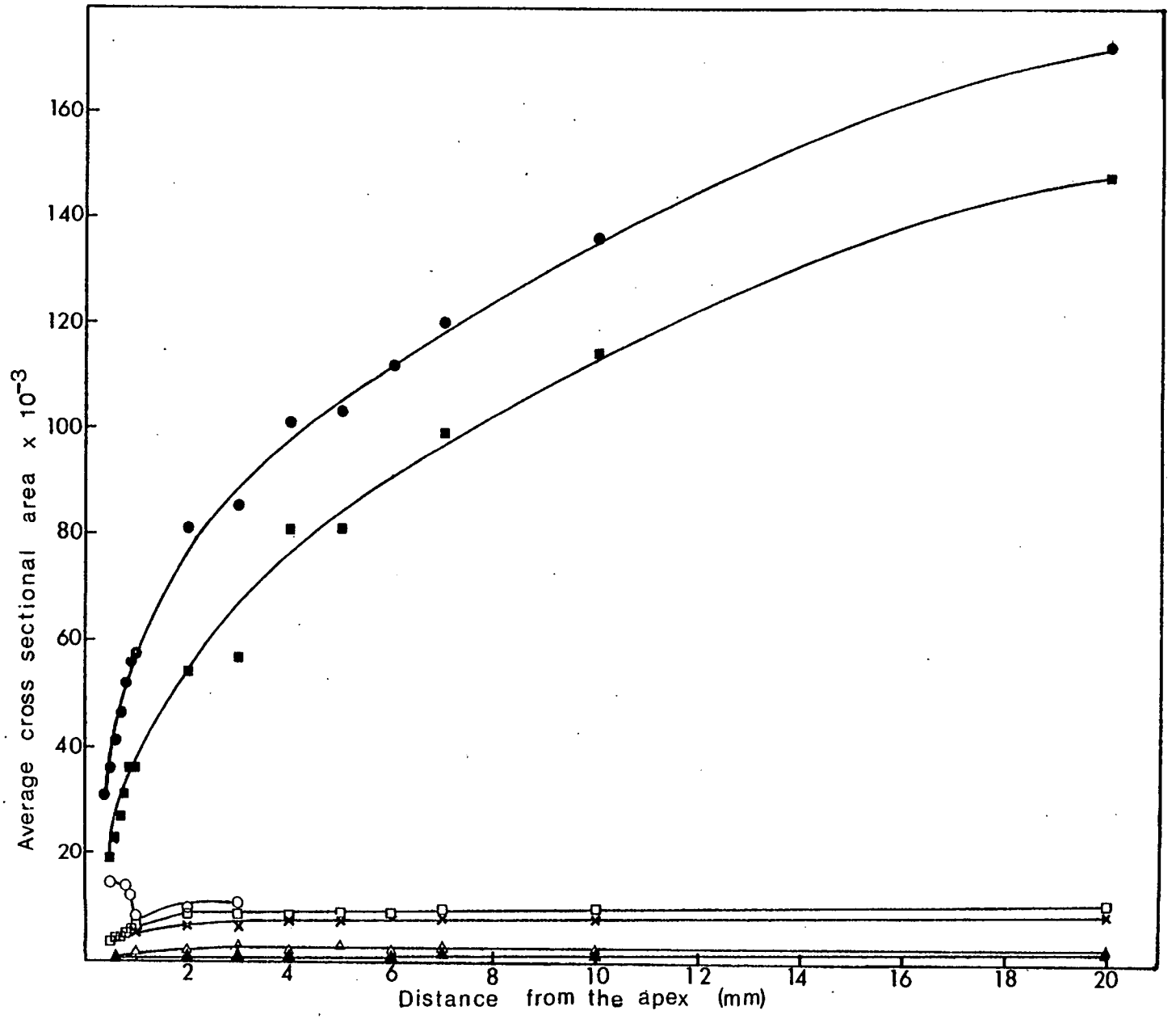

Average cross sectional area x 10^{-3}
160
140
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
Distance from the apex (mm)

Fig. 4:1:3.

the apex in limited meristematic areas during active lateral root formation.

Tissues may be recognised as they emerge at the apex largely by characteristics of cell shape and size, which are generally thought to reflect differences in frequency and plane of cell division in relation to the growth rate of the zone. Cell division and growth characteristics were therefore examined in further detail.

(i) The pattern of cell proliferation in the root tip

a) The apical meristem: Introduction

Most of the cells that constitute the primary root originate within the meristem in the apical millimetre. This zone is shown in median longitudinal section in figure 4:1:1.

At the extreme apex of the root the root cap can be seen to surround the apex of the root proper, with its meristematic zone, the calyptrogen, lying immediately distal to the quiescent centre.

The quiescent centre lies about 420μ from the tip of the root cap between the tip of the procambial cylinder and the root cap columella, thus demarcating the boundary between the growing root and the root cap. The cells in this zone are small, isodiametric, and have a high nuclear/cytoplasmic ratio. However, as the quiescent centre merges into and may include part of the procambium, cortex and root cap, it is difficult to distinguish these cells without autoradiography of sections incubated with radioactively labelled thymidine. On autoradiographs they are observed as a region of a slow rate of DNA synthesis (Clowes, 1959).

The quiescent centre was found by Clowes to be a group of cells with very low meristematic activity (Clowes, 1959, 1961). Its location corresponded to the 'minimal construction centre' (Clowes, 1954), the zone that provided the minimum number of cells required, from anatomical considerations, to produce the pattern of cell files making up the root. As cell division was so infrequent in this zone, the meristematic cells lying on the periphery of this zone were suggested by Clowes (1954, 1961) to be the initials from which all cells of the root were derived.

However, Barlow and MacDonald (1973) have found that the average duration of the cell cycle in the cortex and stele is 0.1- 0.125 that of cells of the quiescent centre, due largely to variation in G_1 phase. Cells are thus continually displaced on the margin of the quiescent centre, and Barlow (1976) has suggested that it may be considered as a group of 'founder' cells that are displaced slowly by division, becoming more actively meristematic 'initials' on the periphery. The derivatives of these initials may themselves divide, eventually ceasing division although they may continue to synthesise DNA and thus become polyploid. The 'initial' cells are slowly and continually replaced by divisions in the quiescent centre. These divisions contribute cells to both the root cap and the root proper.

Cells do not slide past each other in the root apex, therefore growth/unit length of root occurs at the same rate in all cells at the same distance from the meristem. It therefore appears that as there are no abnormally large cells in the meristem, no cell enters a non-proliferative phase within the meristem with the exception of the quiescent centre which also has a very low growth rate. However, frequency and rate of cell division as measured by the duration of the mitotic cycle, and its constituent phases, cell doubling time and rate of cell proliferation, all vary within the meristem, with characteristics according to cell type and area within the meristem (Erickson and Sax, 1956; Webster and MacLeod, 1980; Barlow, 1973, 1983; Barlow and MacDonald, 1973; MacLeod, 1976; Torrey, 1965; Jensen and Kavaljian, 1958).

b) Cell division in the apical meristem of pea root

Cell proliferation in the root apex is difficult to analyse as all methods of estimation have limitations. However, the patterns of cell numbers in different tissues in transverse section, frequency of mitotic figures, cell size and shape were all found to support the view that the frequency and plane of cell division vary throughout the apex according to tissue and position within the meristem.

As may be observed in figures 4:1:1 and 4:1:4, division in metaxylem cells decreases earlier than in adjacent outer procambial

Figure 4:1:4. The apical meristem of *Pisum sativum*

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 C ii. Transverse sections were cut at a) 75μ, b) 175μ, c) 275μ, and d) 500μ from the apex of the root proper, and were stained with toluidine blue for observation under the light microscope.

It can be seen that cells have already developed very clearly different characteristics according to transverse position within 75μ of the distal boundary of the root proper (figure a). Cortical cells (C) have expanded rapidly in cross-sectional area, and contain large numbers of relatively large vacuoles. Very few divisions (d) may be observed in this tissue at this stage. Procambial cells are smaller, reflected in the large numbers of mitotic figures. Central procambial, or metaxylem, cells (MX) already contain fewer mitotic figures and have a slightly higher vacuome content than adjacent proto-phloem (PH) and protoxylem (PX) cells. Mitotic figures are most frequent in outer procambial cells, in which the triarch pattern of phloem and protoxylem is becoming evident by 75μ from the apex of the root proper. Pericycle (P) cells on the boundary of the procambial cylinder are already larger in cross-sectional area than adjacent procambial cells, and have relatively few mitotic figures. The immediately adjacent endodermal cells (EN) are likewise becoming distinguishable by their low radial/tangential ratio. These patterns become increasingly evident throughout the apical meristem as shown in figures b-d and figure 4:1:19.

By 175μ from the boundary of the root cap and root proper (figure b)), mitotic figures have become less prominent, while cell cross-sectional area tends to reflect the former and present frequency of cell division. For example mid-cortical and metaxylem cells, which cease division at a very early stage in the meristem are relatively large. Occasional mitotic figures may still be observed in the inner cortex and pericycle, but remain most frequent in the outer procambial cells. As can be seen in figures c and d, mitotic figures continue to be observed in outer procambial cells further from the apex than in other cell types.

Tissues may therefore be clearly distinguished within the apical meristem in transverse section, and all major tissues of the stele become evident within this region. Within the stele phloem arcs may be discerned not only by differential vacuolation, but also by their characteristic patterns of cell shape and size. The arcs are delimited at their inner boundary by the curve of highly vacuolated future vascular cambial cells which are elongated in the plane of the curve, and at the outer boundary by radially elongated cells. Central phloem cells tend to be small, more isodiametric and irregular in shape. Single proto-phloem sieve elements (SE) arise adjacent to pericycle cells at the centre of the phloem arcs, appearing successively in each phloem arc. Metaxylem is clearly discernible from protoxylem by rapid expansion and vacuolation.

a) x 315; b) x 315; c) x 315; d) x 315

Fig. 4:1:4.

cells. This is reflected in a lower frequency of mitotic figures and an apparently more rapid rate of expansion (figures 4:1:4 and 4:1:5). Similarly, cells of the mid-cortex cease longitudinal division nearer to the apex than adjacent inner or outer cortical cells, and this again is reflected in a lower frequency of mitotic figures and a more rapid increase in cross-sectional area (Fig. 4:1:4, 4:1:5, and 4:1:19). Procambial cells undergo fewer transverse divisions from an early stage in the meristem than cortical cells, and thus are more elongated (Fig. 4:1:1).

Beyond 1 millimetre from the root tip, increase in cell number is much reduced (Fig.4:1:2) and mitotic figures are much less frequently observed. Mitotic figures in the apical meristem are, however, observed further from the tip in outer stelar cells than in metaxylem or cortical cells (Fig.4:1:4e).

The observation that cell division ceases early in metaxylem and mid-cortical cells has been noted in a number of different types of root (Hejnowicz, 1959; Heimsch, 1951; Hagemann, 1956; Esau, 1965). Mitotic figures have also been found to be more frequent further from the root tip in stelar than cortical cells in other species (Jensen and Kavaljian, 1958; Hagemann, 1956; Bucknall and Sutcliffe, 1965). These consistent patterns of variation indicate that frequency of division has characteristics according to each cell type in the meristem which are common to these cell types regardless of species from which they are derived, or of growth conditions.

c) Cell proliferation proximal to the apical meristem

Cell proliferation outside the apical meristem is normally disregarded as estimates of cell number are generally made on a cell number/section basis. These studies show a decrease in cell number/section from the meristem owing to cell elongation which reduces the number of cells/unit length of root. However, an analysis of cell number in individual tissues from anatomical studies of successive transverse sections of pea root indicated that longitudinal cell divisions continued for the entire 20mm (Fig.4:1:2).

Longitudinal cell divisions continued outside the apical

Figure 4:1:5. Alterations in cell cross-sectional area in different tissues during differentiation in the root apex.

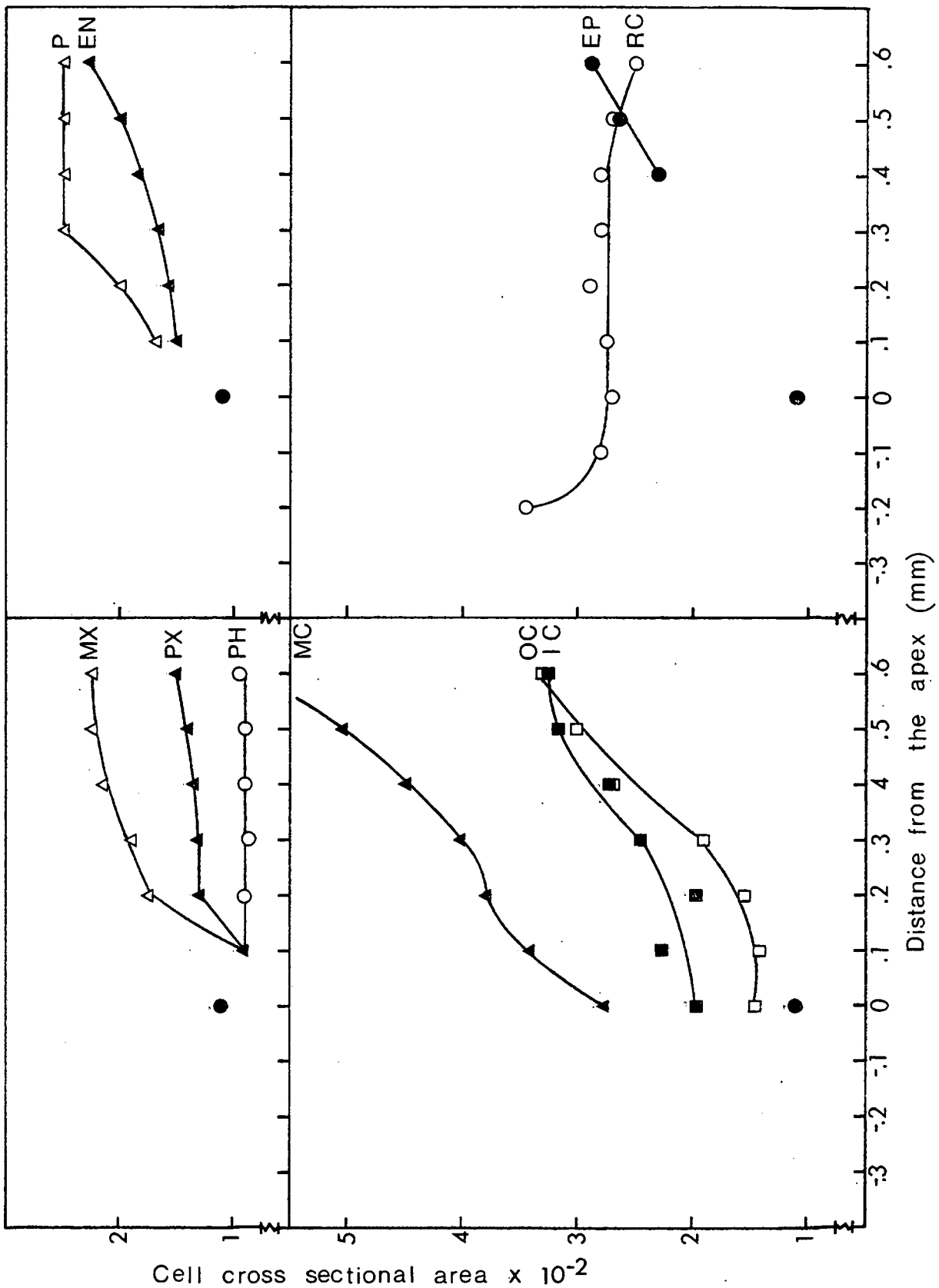
Pea root tissue was prepared for observation under the light microscope as described in chapter 2 section 2 C ii, and serial transverse sections were obtained at 100μ within the apical millimetre and at one millimetre intervals thereafter. Cell dimensions were then measured with the aid of a micrometer eyepiece. Tissues shown in figures a and b are metaxylem (——△——MX), protoxylem (——▲——PX), phloem (——○——PH), pericycle (——△——P), endodermis (——▲——EN), mid cortex (——▲——MC), outer cortex (——□——OC), inner cortex (——■——IC), epidermis (——●——EP), root cap (——○——RC) and procambium (——●——).

a) Cell cross-sectional area in the apical millimetre of pea root.

b) Cell cross-sectional area in the apical 20mm of pea root.

Fig. 4:1:5.

a


P
EN
EP
RC
MX
PX
PH
MC
OC
IC
Distance from the apex (mm)
Cell cross sectional area x 10^{-2}

b

Distance from the apex (mm)
0 2 4 6 8 10 12 14 16 18 20
Cell cross sectional area x 10^{-2}
0 4 8 12 16 20
RC
EP
EN
P
IC
OC
MC
PH
MX
PX
0 2 4 6

meristem as can be observed by the increases occurring in cell numbers in single layers of different tissues in transverse section (Fig.4:1:2). The increase in cell number is provided largely by divisions in inner cortical, pericycle and endodermal cells, which appear, from observation of transverse sections, to be confined to the areas adjacent to the protoxylem poles (Fig.4:1:6). It should be noted that it is not possible to use mitotic index as an accurate indicator of frequency of cell division as it is known that the length of mitosis varies throughout the apical meristem in pea roots. (Barlow and MacDonald, 1973). However, to quite a large extent mitotic frequency in pea root was found to reflect frequency of division as indicated by increase in cell number, and was therefore a useful indicator of areas within a zone undergoing more frequent cell division. Furthermore, in endodermal, pericycle and inner cortical layers the pattern of cell shape and size in the layers adjacent to protoxylem cells becomes disrupted, cells being smaller and less characteristically shaped than cells adjacent to phloem. This, coupled with the observation that mitotic figures were only observed in the cells adjacent to protoxylem, indicated that divisions were confined to these zones.

By 1mm from the tip (Fig. 4:1:2) the cell number in endodermal and pericycle cells continued to increase, although divisions decreased rapidly in cells of other tissues, with a later decrease in the outer stele than in cortical or inner stelar cells (figure 4:1:4e). The pattern of cell shape has already become disrupted in the endodermal and pericycle cells adjacent to the protoxylem, the cells being more densely staining and less tangentially elongated reflecting the occurrence of anticlinal divisions in these zones (Fig.4:1:4e and 4:1:6a). Mitotic figures become more frequent 2.5-4mm from the tip, when proliferation of inner cortical cells also commences, mitotic figures being most prominent at around 4mm from the apex. Endodermal and pericycle divisions continue until about 7mm from the apex, although cells in the inner cortex, which will include cells derived by periclinal division of pericycle and endodermal cells, continue to divide (Fig.4:1:2).

Figure 4:1:6. Meristematic areas proximal to the apical meristem in *Pisum sativum*

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in araldite as described in chapter 2 section 2 C ii. Light micrographs shown are transverse sections cut at a) 2mm, and b), c) 4mm from the root apex, and stained with toluidine blue.

Cells of the endodermis (EN), pericycle (P), and inner cortex (IC) opposite the protoxylem arcs (Px) remain more densely staining and less vacuolate than adjacent cells of these tissues. This may be clearly seen in figure a. These cells remain meristematic.

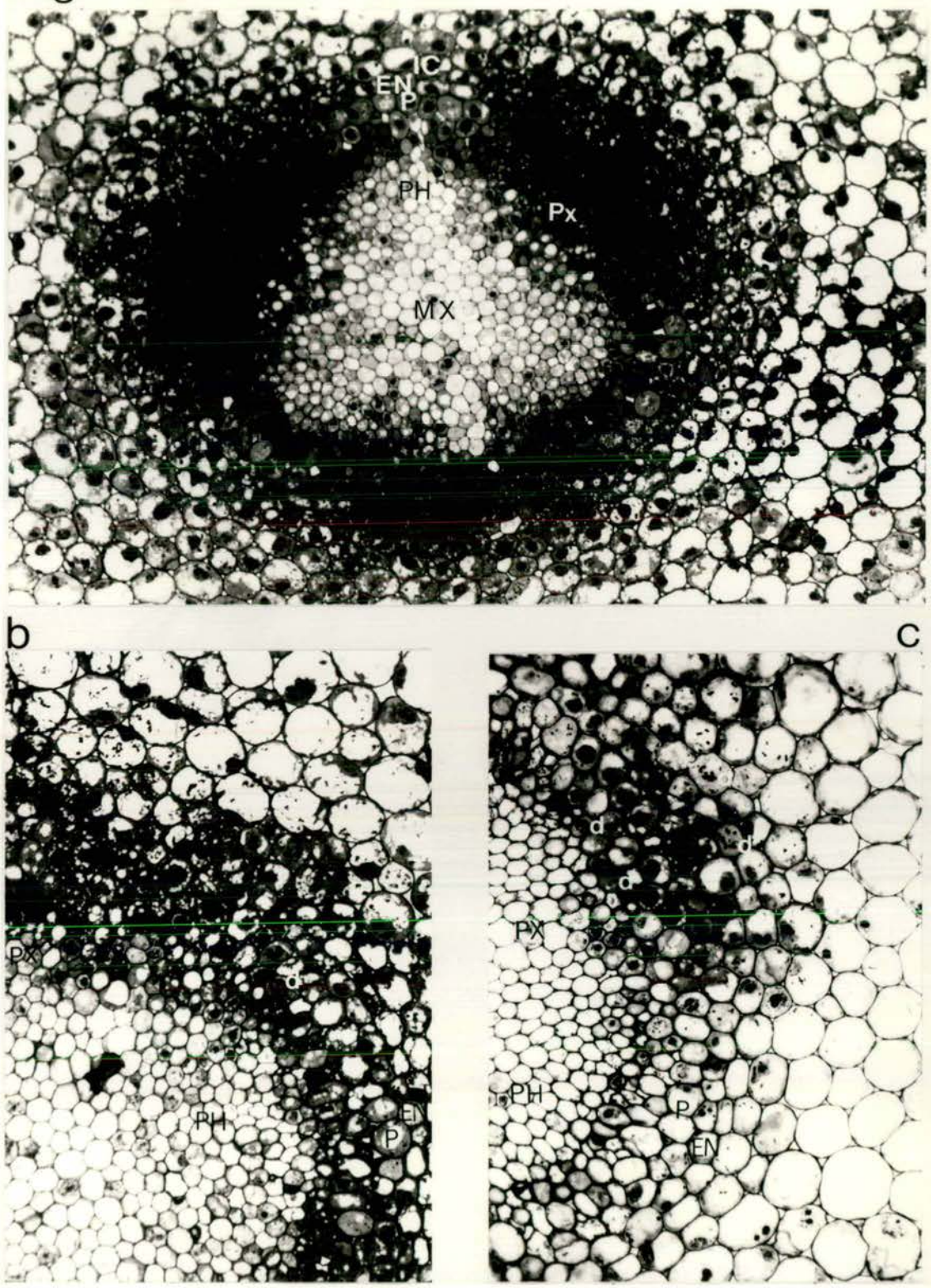
Mitotic figures (d) are particularly frequent 3.5-4mm from the tip (figures b and c), disrupting the pattern of cell shape and size locally.

a) x 213

b) x 315

c) x 315

Fig. 4:I:6.



It has been noted in a number of species that cells adjacent to the protoxylem in triarch roots remain meristematic in one or more of the layers of endodermis, pericycle and inner cortex (Libbenga *et al.*, 1973; Seago, 1973). Lateral roots are generally regarded as being initiated in mature regions of the root (Esau, 1965b). However, as these cells eventually produce lateral roots, it can be seen that lateral root primordia may be initially specified within the apical mm of pea root in accordance with the observations of Seago (1973) for lateral root initiation in *Ipomoea* and with a number of aquatic plants and members of the Cucurbitaceae and Papilionaceae (Esau, 1965b).

(ii) The development of cell shape

a) Orientation of division and its effect on cell shape

Cells are identifiable immediately beyond the quiescent centre as belonging to a particular tissue largely by virtue of cell shape and size. To a large extent, the shape of cells in the apex and in developing lateral root primordia appears to be determined by the plane of cell division, while size is regulated more by frequency of division, rate of elongation and of radial expansion. In relation to this it is interesting to note that not only the frequency of division, but also the predominant orientation of cell division vary between tissues and within a tissue according to both position within the tissue and stage of development.

One of the main examples of preferential polarity of division is that of periclinal divisions in the inner cortex which give rise to endodermal cells of very low radial/tangential ratio (Fig.4:1:7a). Within 400μ of the quiescent centre however, the ratio begins to rise, reaching a final ratio of 1 when cell division has almost ceased by 7mm (Fig.4:1:7b). The increase in ratio is initially due to an increase in radial (Fig.4:1:8a) but not tangential (Fig.4:1:9a) diameter. As the circumference of the endodermis rises (Table 4:1:2) and cell number increases in this layer (Fig.4:1:2), the change in ratio reflects anticlinal division in the endodermis and is largely confined to areas adjacent to protoxylem poles. The radial (Fig.4:1:8b) and

Figure 4:1:7. The development of cell radial/tangential ratios in a variety of tissues during differentiation in the pea root apex

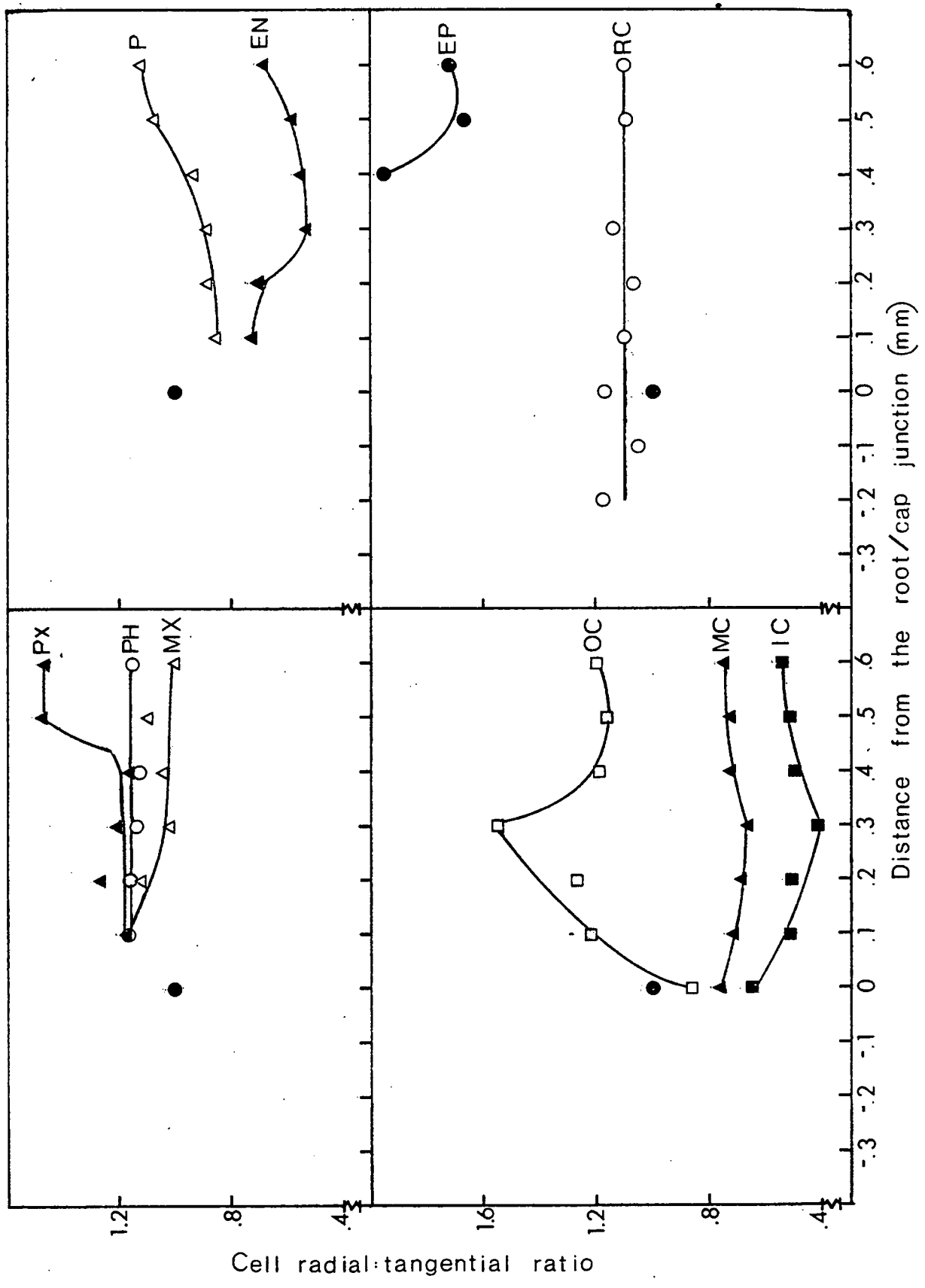
Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were cut at 100μ intervals within the apical millimetre and at one millimetre intervals thereafter. Cell dimensions in different tissues were measured with the aid of a micrometer eyepiece. Tissues measured were metaxylem (——△——MX), protoxylem (——▲——PX), phloem (——○——PH), pericycle (——△——P), endodermis (——▲——EN), mid cortex (——▲——MC), outer cortex (——□——OC), inner cortex (——■——IC), epidermis (——●——EP), root cap (——○——RC), and procambium (——●——).

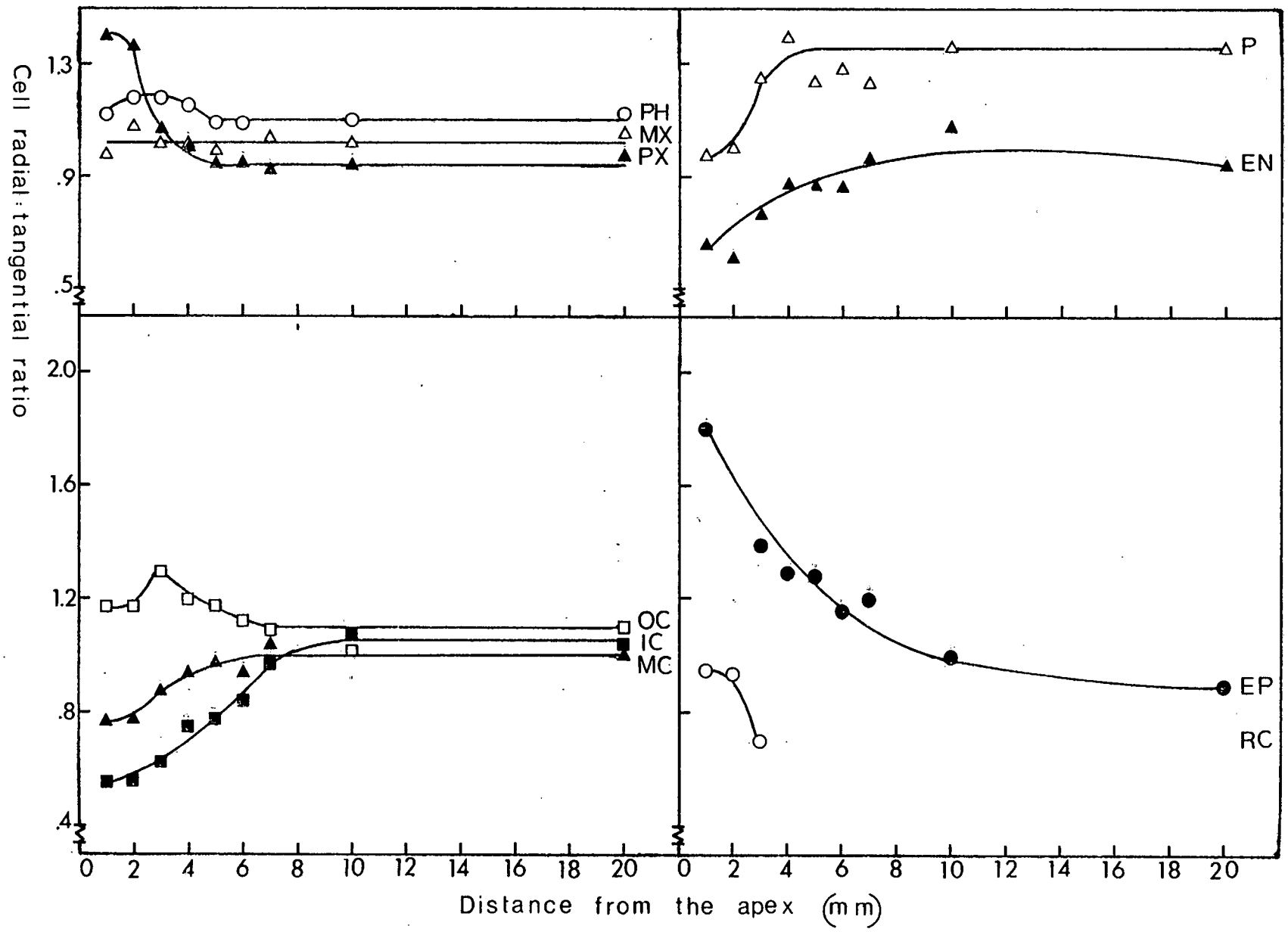
a) Cell radial/tangential ratio within the apical millimetre of pea root.

b) Cell radial/tangential ratio within the apical 20mm of pea root.

Fig. 4:1:7.

a


P
EN
EP
RC
PX
PH
MX
OC
MC
IC
1.2
.8
.4
1.6
1.2
.8
.4
-.3 -.2 -.1 0 .1 .2 .3 .4 .5 .6
Distance from the root/cap junction (mm)
Cell radial:tangential ratio


Cell radial : tangential ratio
1.3
.9
.5
PH
MX
PX
P
EN
2.0
1.6
1.2
.8
.4
OC
IC
MC
EP
RC
0 2 4 6 8 10 12 14 16 18 20
Distance from the apex (mm)

b

Figure 4:1:8. Changes in cell radial diameter in a variety of tissues during differentiation in the pea root apex

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were cut at 100μ intervals within the apical millimetre and at one millimeter intervals thereafter. Cell radial diameters in a variety of tissues were then measured with the aid of a micrometer eyepiece. Tissues measured were metaxylem (——△——MX), protoxylem (——▲——PX), phloem (——○——PH), pericycle (——△——P), endodermis (——▲——EN), mid cortex (——▲——MC), outer cortex (——□——OC), inner cortex (——■——IC), epidermis (——●——EP), root cap (——○——RC), and procambium (——●——).

a) Radial diameters of cells within the apical millimetre of pea root.

b) Radial diameters of cells within the apical 20mm of pea root.

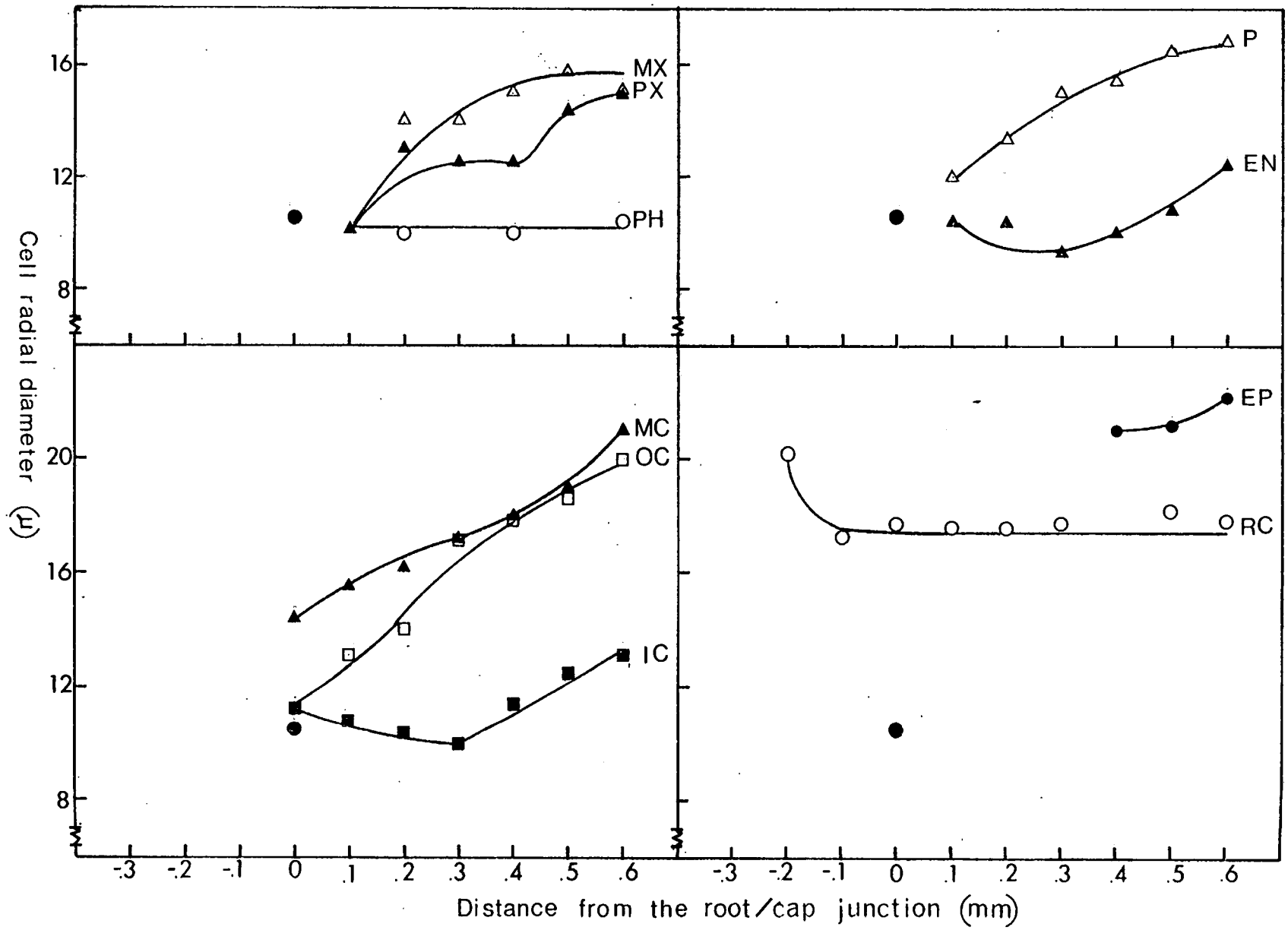
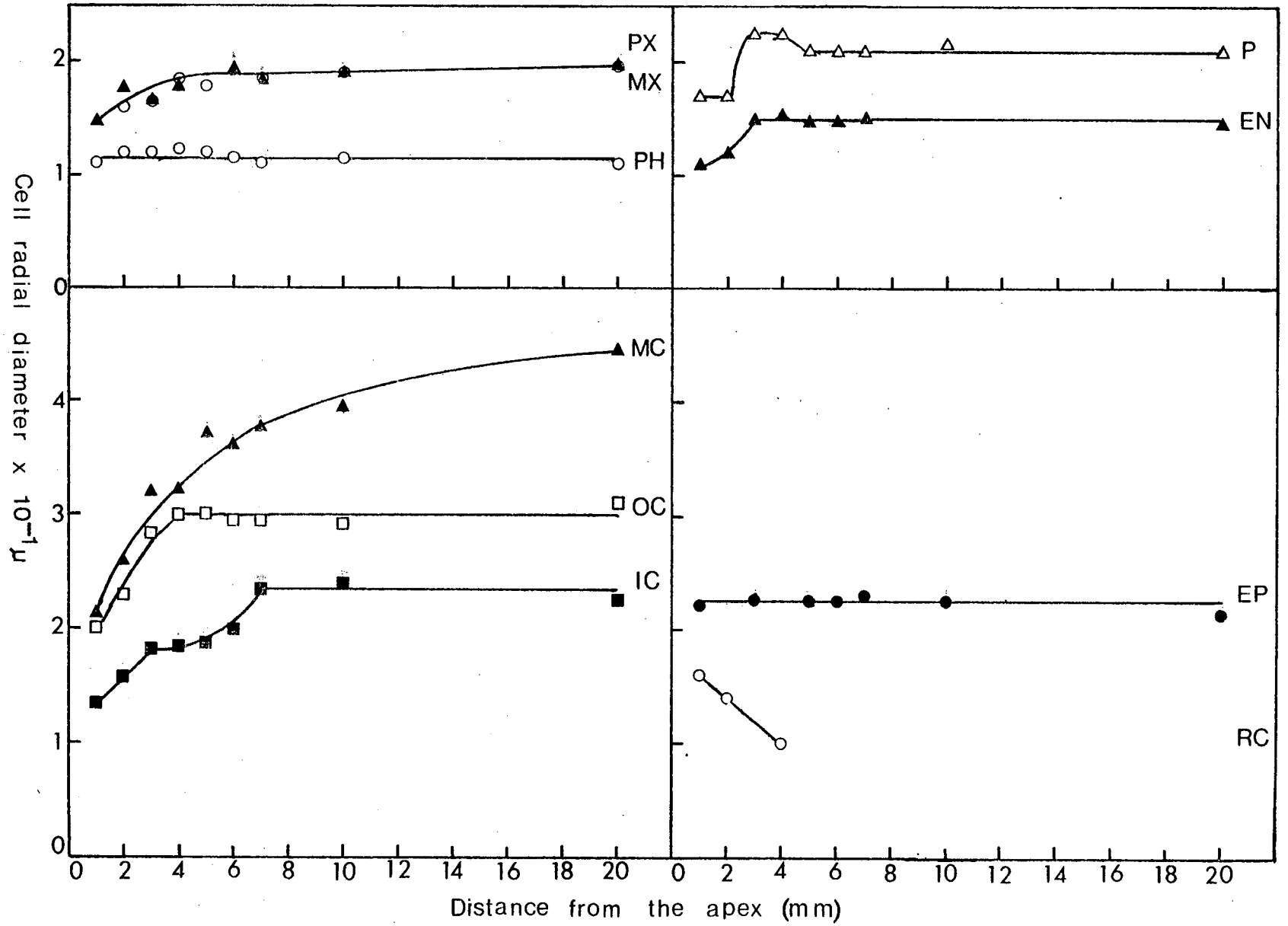

Cell radial diameter (μ)
16
12
8
20
16
12
8
MX
PX
PH
P
EN
MC
OC
IC
EP
RC
-.3
-.2
-.1
0
.1
.2
.3
.4
.5
.6
Distance from the root/cap junction (mm)
a

Fig.4:1:8.


PX
MX
PH
P
EN
MC
OC
IC
EP
RC
Cell radial diameter x $10^{-1}\mu$
Distance from the apex (mm)
0
1
2
3
4
0 2 4 6 8 10 12 14 16 18 20

9

Figure 4:1:9. Changes in cell tangential diameter in a variety of tissues during differentiation in the pea root apex

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were cut at 100μ intervals within the apical millimetre, and at one millimetre intervals thereafter. Cell tangential diameters in a variety of tissues were then measured directly with the aid of a micrometer eyepiece. Tissues measured were metaxylem (—△—MX), protoxylem (—▲—PX), phloem (—○—PH), pericycle (—△—P), endodermis (—▲—EN), mid cortex (—▲—MC), outer cortex (—□—OC), inner cortex (—■—IC), epidermis (—●—EP), root cap (—○—RC), and procambium (—●—).

a) Tangential diameters of cells within the apical millimetre of pea root.

b) Tangential diameters of cells within the apical 20mm of pea root.

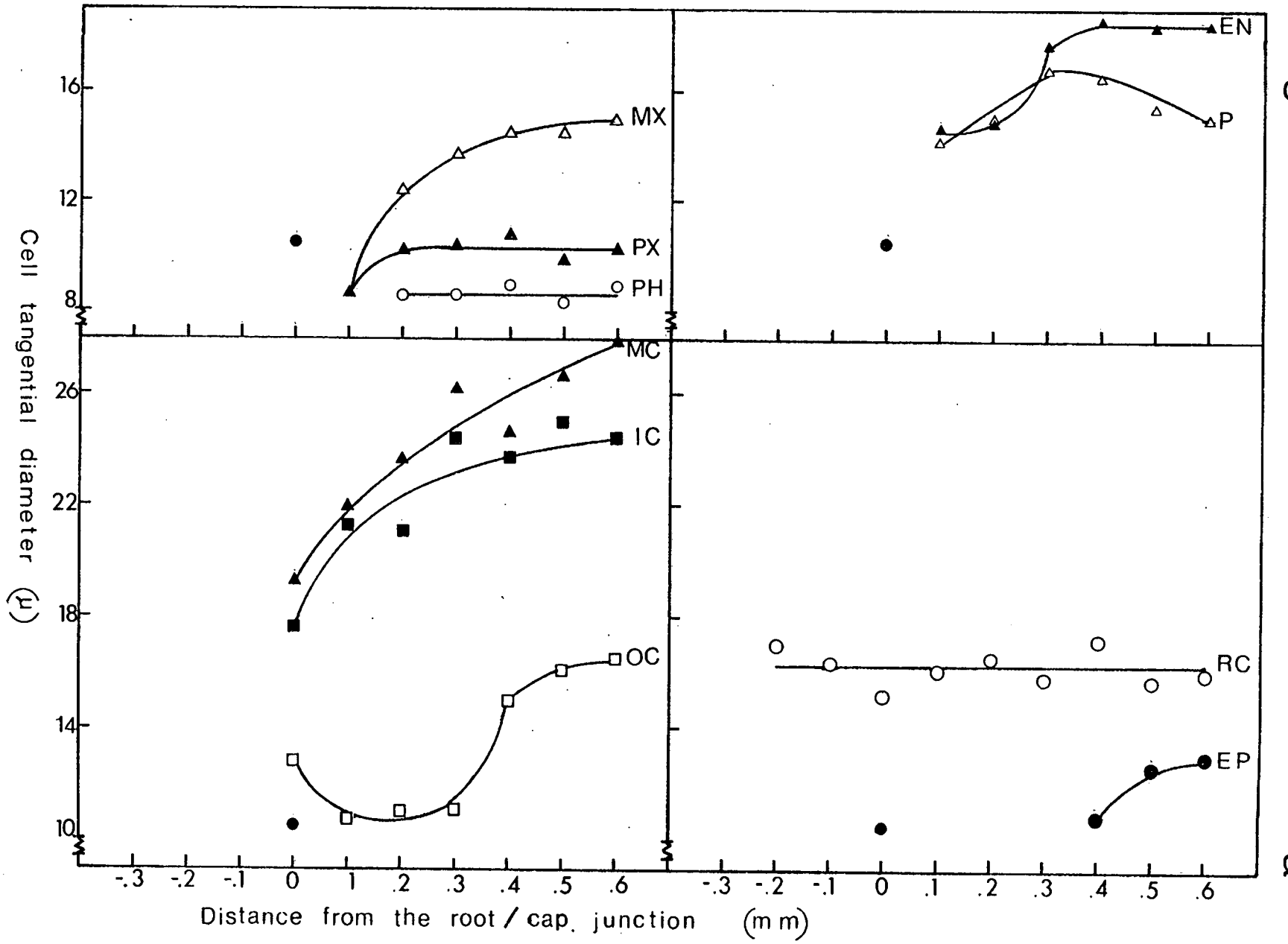

Cell tangential diameter (μ)
16
12
8
MX
PX
PH
EN
P
26
22
18
14
10
MC
IC
OC
RC
EP
-.3 -.2 -.1 0 1 .2 .3 .4 .5 .6
-.3 -.2 -.1 0 .1 .2 .3 .4 .5 .6
Distance from the root / cap. junction (mm)

Fig. 4:1:9. a

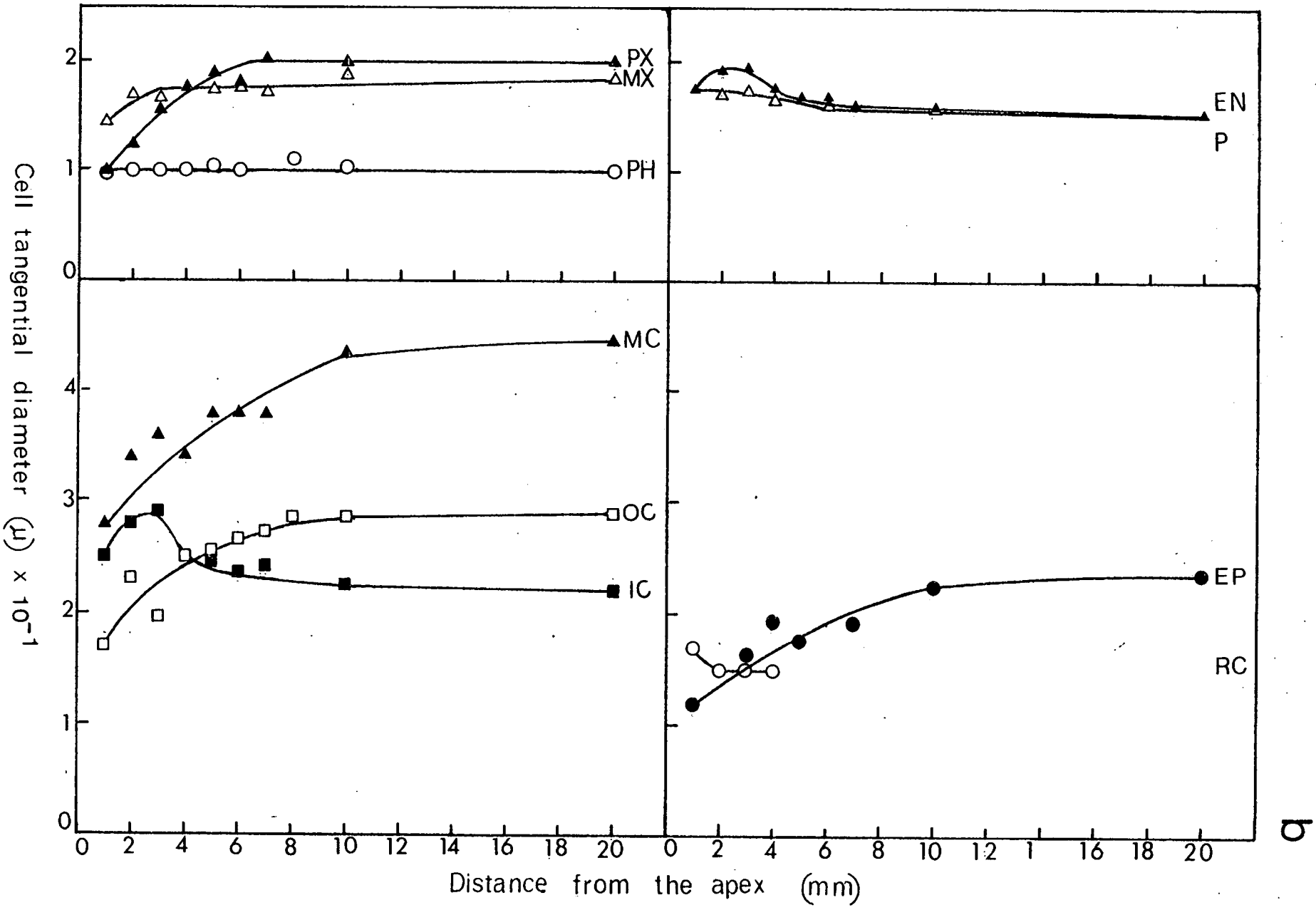

PX
MX
PH
EN
P
MC
OC
IC
EP
RC
Cell tangential diameter (μ) × 10⁻¹
Distance from the apex (mm)
0
1
2
3
4
0 2 4 6 8 10 12 14 16 18 20
b

Table 4:1:2. Developmental changes in tissue boundaries in the pea root apex

Pea roots were prepared for observation under the light microscope, transverse sections were obtained, and the diameters at the boundaries of concentric tissue cylinders were then measured. The circumferences of tissue boundaries were estimated from the diameters, under the assumption that the boundaries were circular.

Tissue boundaries estimated were the root cap/epidermis (RC/EP), outer cortex/epidermis (OC/EP), endodermis/inner cortex (EN/IC), pericycle/endodermis (P/EN), and the stele/pericycle (ST/P) boundaries.

Distance from the apex	Root	Tissue boundaries (μ) EP/RC	OC/EP	EN/IC	P/EN	ST/P
0[1]	1980					
100[1]	2140	1660		650		
200[1]	2430	1870		780	720	660
300[1]	2430	2030		880	810	720
400[1]	2580	2190		960	880	800
500[1]	2640	2350		1020	950	850
1[2]	2680	2500	2370	1080	1000	900
2[2]	3200	3000	2870	1220	1150	1030
3[2]	3280	3080	2940	1230	1150	1030
4[2]	3570		3420	1260	1170	1040
5[2]	3590		3430	1270	1190	1070
6[2]	3750		3610	1280	1180	1080
7[2]	3900		3750	1300	1210	1110
10[2]	4130		4000	1310	1240	1110
20[2]	4660		4530	1400	1310	1190

1. Distance from the root cap/root proper junction, in micrometres.

2. Distance from the root tip, in millimetres.

tangential (Fig.4:1:9b) cell diameters then both rise as division slows down but the endodermal circumference continues to rise. Beyond 3mm the cell radial diameter remains constant while tangential diameter decreases until 7mm from the apex, reflecting almost complete cessation of increase in circumference of the layer but continuing anticlinal divisions in the zone adjacent to protoxylem. Although the average cell tangential diameter and cell cross-sectional area remain constant after about 10mm from the apex, a few (about five) more anticlinal divisions may occur by 20mm as is indicated by a slight increase over this zone in the circumference of the layer.

The radial/tangential ratio of cells of the pericycle layer when it becomes discernible by 100μ from the quiescent centre is higher than that for endodermal cells, although it is also below 1 (Fig.4:1:7a). As the circumference of the layer increases (Table 4:1:2) the ratio rises gradually due to a rapid rise in average cell radial diameter to 3mm from the apex (Fig.4:1:8a and b), and a slower rise in tangential diameter to 300μ from the quiescent centre after which it remains at a constant value (Fig.4:1:9a and b), thus allowing an increase in the ratio to 1.4 by 4mm from the tip of the root. As cell number increases throughout this zone, the change in radial/tangential ratio again appears to reflect the far greater frequency of anticlinal than periclinal divisions.

Similarly, the very low radial/tangential ratio of inner cortical cells in the meristem reflects periclinal divisions in the meristem to produce endodermal cells. Cell division is not observed between 1 and 3mm from the apex, and as the circumference of the layer increases both the radial and tangential diameters therefore increase to 3mm. The radial/tangential ratio increases after 3mm due to a reduction in average tangential diameter as inner cortical cells near the protoxylem poles go into division, mainly in the anticlinal plane. After 8mm when divisions are less frequent and there is little further increase in circumference of the layer, the radial/tangential ratio remains roughly constant indicating that the continuing increase in cell number in this layer is due to both anticlinal and periclinal division.

Phloem cells have the same average cross-sectional area and radial/tangential ratio throughout the apical 20mm. However, the pattern of cell division is highly characteristic of the position within the tissue and becomes evident by 200μ from the quiescent centre/cap junction (Fig.4:1:4d and e). The walls of cells on the inner boundary of the phloem arc describe the arc, and cells are elongated parallel to this boundary. These cells will become the vascular cambium. By contrast, cells on the outer boundary adjacent to the pericycle are elongated radially. All boundary cells tend to be large, while central phloem cells tend to be much rounder in shape and decrease progressively in size from the inner zone to rows of very small cells adjacent to the outer boundary layer (Fig. 4:1:4 and 4:1:19c). Cell divisions are observed in phloem, particularly in metaphloem as far as 2mm or more from the apex. These appear to be largely divisions of metaphloem to form sieve elements and companion cells, and are frequently unequal divisions. The increase in cell number does not appear to reduce the average cross-sectional area of phloem cells as protophloem sieve elements are often crushed and reduce in size (Fig.4:1:17) or collapse.

It is not clear to what extent polarity of growth or differential growth rate might contribute to this pattern; however, it seems likely that the major influences in development of cell shape are the orientation of cell division, localised differences in the frequency of division, and unequal division at least in the formation of sieve elements and companion cells.

Procambial and cortical cells also may be identified adjacent to the quiescent centre by a greater relative frequency of transverse divisions in the cortex than in the procambium, resulting in more elongated procambial cells (Fig.4:1:1 and 4:1:11).

b) Change in cell shape in the absence of cell division

The patterns of cell shape and size appear generally to reflect the frequency and orientation of cell division in the apical meristem and developing lateral root primordia. Thus, as tissues are recognisable immediately adjacent to the quiescent centre largely by characteristics of cell shape and size, the

characteristics of cell division are largely responsible for divergence of tissues at the structural level at the apex. However, as change in radial/tangential ratio alters without cell division in maturing protoxylem, mid-cortical and epidermal cells (Fig.4:1:2 and 4:1:7), it appears that polarity of growth or differential rate of growth may also affect development of cell shape.

Increase in cross-sectional area of the root tip must have a radial gradient of expansion, otherwise tensions will be created, and the area occupied by intercellular spaces will increase, or splitting of the root will occur. Thus, the inner zones must expand more slowly than the outer zones. As the cross-sectional area of pea root increases throughout the apical 20mm (Fig.4:1:3), expansion throughout this zone has to be coordinated at least to some extent. It is therefore interesting to note that the proportion of root cross-sectional area occupied by different tissues varies throughout the root tip (Fig.4:1:10), in some instances independently of the production of new cell layers by division.

It can be seen from figure 4:1:10 that the cortex occupies an increasingly large proportion of the root particularly in the apical 6mm, due almost entirely to expansion of mid-cortical cells (Fig.4:1:5) which additionally have a higher degree of radial than tangential expansion (Fig.4:1:7). As cell number does not increase within this group of cells (Fig.4:1:2), this represents a radial polarity of growth in cells of the mid-cortex. The increase in area occupied by intercellular spaces in cortical cells may perhaps be a reflection of this polarity.

As cortical tissue increases as a proportion of the root there is a corresponding decrease in the proportion of cross-sectional area occupied by the epidermis and by the stele (Fig. 4:1:10). Epidermal cells do, however, expand at the same time as the cortex expands (Fig.4:1:5), the cells becoming tangentially elongated (Fig.4:1:7b) due to expansion in the tangential but not the radial plane (Fig.4:1:8b and 4:1:9b). These observations indicate that the cortex may be exerting mechanical pressure on the epidermis, and that the epidermis does not expand sufficiently to maintain its cross-sectional area as a proportion of the root. As a result, the epidermal cells become stretched

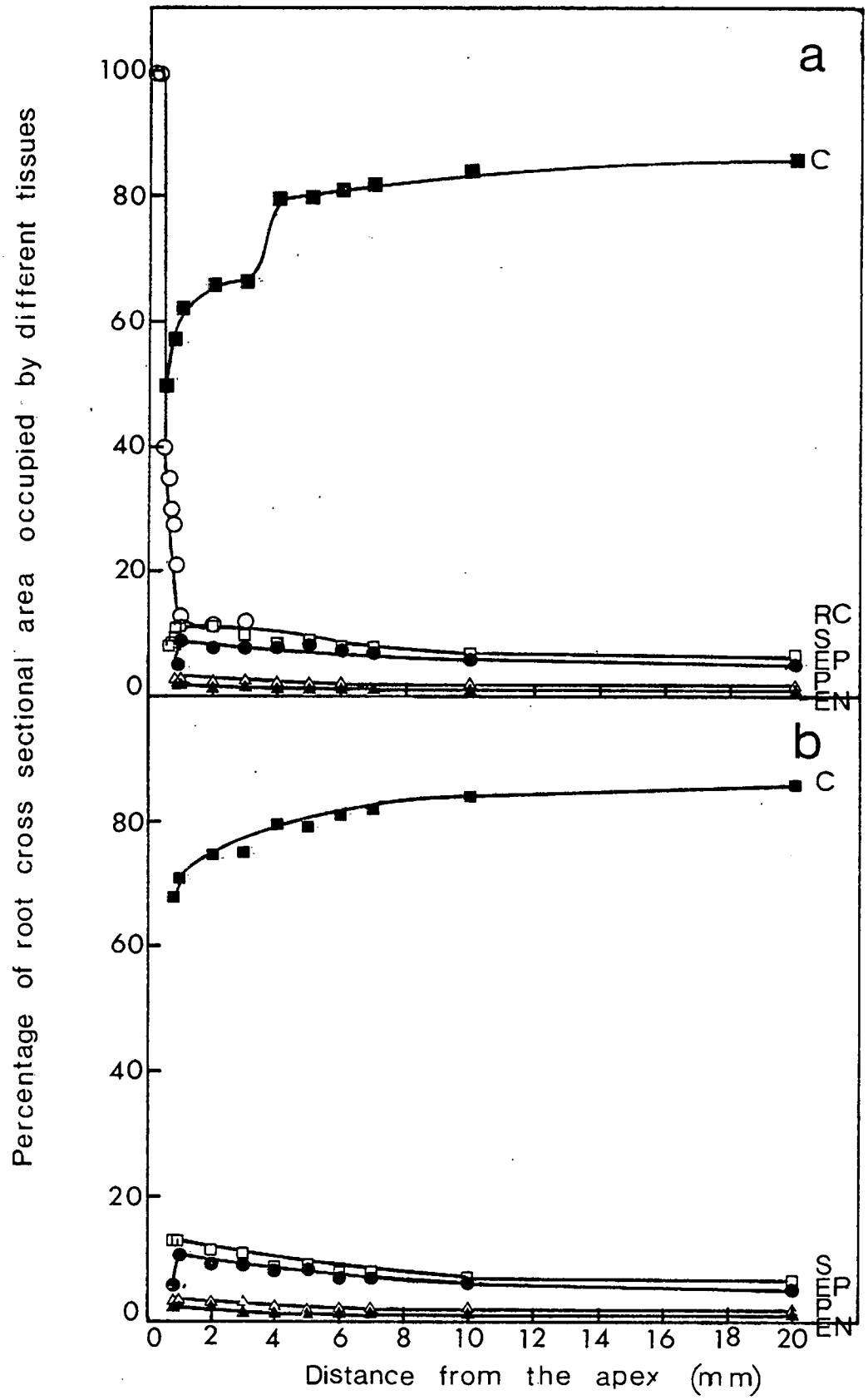
Figure 4:1:10. Developmental changes in the proportions of the cross-sectional area of the root occupied by different tissues

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were then cut at 100μ intervals within the apical millimetre, and at one millimetre intervals thereafter. The inner and outer diameters of tissue boundaries were measured, and cross-sectional area of tissues then estimated under the assumption that the tissue boundaries were circular. Tissues examined include the root cap (—○—RC), epidermis (—●—EP), cortex (—■—C), endodermis (—▲—EN), pericycle (—△—P), and the stele (—□—S).

a) Proportions of the root cross-sectional area occupied by different tissues, inclusive of the root cap.

b) Proportions of the root cross-sectional area occupied by different tissues, exclusive of the root cap.

Fig. 4:1:10.


a
b
C
RC
S
EP
P
EN
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
Distance from the apex (mm)
Percentage of root cross sectional area occupied by different tissues

tangentially. Alternatively, rapid tangential expansion of epidermal cells might produce tensions in the cortical cells as the circumference of the outer cortical boundary increases. This might induce rapid cortical expansion with an increasing area occupied by intercellular spaces as cell expansion does not keep pace with the increase in area required. Radial polarity of growth might then be a reflection of radial stretching induced by epidermal expansion. Although it is not clear which layer, if any, initiates these tensions, Barlow *et al* (1982) have suggested that cortical cells are specialised to generate high osmotic pressures within their vacuome in connection with the development of an ion uptake mechanism; the resulting increase in size possibly forcing a change in expansion of adjacent cells. Burström (1971) has also observed that the root is under longitudinal stress, the cortex appearing to be restricted by slower growth of the inner cylinders. It may therefore be possible that the growth rate of the cortex is higher than that of the inner cylinders and possibly of the epidermis; either due to limited extensibility of the wall in the longitudinal plane in stelar cells, to relative ease of radial expansion in cortical cells, to restriction of growth in the longitudinal rather than the radial plane in cortical cells or to a combination of these. The increased growth of the cortex and polarity of growth may therefore reflect a higher growth rate in conjunction with limitations on the direction of expansion.

Similarly, although the stele decreases as a proportion of the root after the apical millimetre due to the relatively fast expansion of cortical cells, it does continue to expand slowly throughout the entire apical 20mm (Fig.4:1:3). Again, differential polarity of expansion occurs. The central metaxylem cells expand, and a pressure would be exerted on the outer stele if this zone did not increase tangentially. As intercellular spaces do not appear in the outer stele, this zone must therefore expand to accommodate the stress. Phloem tissue does not appear to expand to any great extent, and the increase in area is provided largely by an increase in tangential expansion of protoxylem cells (Fig.4:1:9b). Again it is not clear which zone, if any, initiates the change in tension, and it may be

for example that tangential protoxylem expansion is induced as a result of localised tension caused by divisions in adjacent layers during the initiation of lateral root primordia. The resulting increase in tangential protoxylem expansion might induce metaxylem expansion. Alternatively, differential rate and polarity of expansion may be programmed rather than induced.

Although any polarity of growth is obscured early in root development by cell division, protoxylem, mid-cortical and epidermal cells all undergo a change in radial/tangential ratio after longitudinal divisions have ceased in these cells, demonstrating polarity of expansion. It appears to either cause or be the result of mechanical pressure on adjacent cells.

It therefore appears that cells do not undergo a synchronous increase in expansion with a smooth radial gradient of expansion. To some extent growth does reflect the radial position of a tissue. However, different tissues and different zones within a tissue have characteristic rates and polarity of growth which may also vary throughout the apical 20mm.

Compensation for tension induced by cell expansion in adjacent cell layers is not completely attained, and this may result in an increase in area occupied by intercellular spaces or in relative alterations in the proportions of root area occupied by the tissues. Compensation for tension within the root may be achieved by alteration in growth rate or polarity of growth of adjacent cells, or by alteration of the area occupied by intercellular spaces; the response depending on the tissue involved.

It therefore appears that a negative and flexible coordination rather than synchrony is achieved between cells to maintain root shape in the transverse plane. This is different from the case of elongation in which synchrony of elongation is achieved between cells of different tissues involving widely different total growth/cell and relative elongation growth/cell, although not of rate of elongation/unit length of cell, indicating that there may be a general limiting factor such as wall extensibility. Expansion in cross-sectional area also differs from elongation in that the region of radial expansion does not correspond exactly to the region of cell elongation although they do overlap as most radial enlargement occurs nearer the apex than the area

of maximum elongation (Fig.4:1:3 and 4:1:11). Radial expansion may therefore be under a different control mechanism from elongation, or may involve additional superimposed control mechanisms, for example for sensing tension in the plasmalemma with a corresponding growth response. Expansion induced by this mechanism might then proceed within any limits imposed by extensibility and the limits of the capacity of the tissue to respond by altering growth. Alternately, differential expansion may be a result of different growth rates with differential capacities for radial and tangential wall expansion.

(iii) Cell expansion in the apex

a) Rate of expansion

The rate of cell elongation is most commonly measured by marking points along the root and observing displacement of the marks with time. These measurements indicate that total elongation/section is highest a few millimetres beyond the meristem, and is virtually complete by 10mm in pea root (Chaly and Setterfield, 1975). This has led to the concept of an 'elongation zone' beyond the meristem stretching for a few millimetres along the root.

However, these measurements are normally obtained using relatively large segments of about 1mm in length, which is perhaps not appropriate in the apical meristem as roughly half the length of the apical millimetre consists of root cap cells which do not contribute to the developmental sequence of the root proper. Furthermore, the rate of growth/cell or /unit length of root is perhaps a more useful measure of growth for comparison between different zones than total elongation over large sections which are comprised of cells differing greatly in length within the section and between sections.

The relative elongation rate (rate of elongation/unit length of root) has been measured by Goodwin and Stepka (1945), Erickson and Sax (1956) and Goodwin and Avers (1956) by measuring increases in very small sections of the root. They observed that there appears to be a zone of more rapid increase

in rate in the meristem beyond the quiescent centre followed by a decrease towards the base of the meristem, before rising again to reach a maximum further back in the root. Goodwin and Stepka (1945) found that in *Phleum* the relative rate of elongation was highest in the basal portion of the meristem, and just beyond. Hejnowicz (1959) on the other hand found a constant growth rate within the meristem. Thus, it appears that there may be an additional zone of relatively high elongation rate within the meristem that could not be detected using larger sections.

It should also be noted that rate of elongation and rate of growth/cell will vary considerably between tissues at the same level owing to the differences in cell length and cell volume. Rate of increase in volume/unit length of root or /cell may also have different values from those for rate of elongation as the diameter of the root proper (minus root cap) increases two-fold within the first 500μ from the quiescent centre.

b) Cell expansion

I. Elongation

Comparison of mean cell volume at increasing distances from the apex is commonly made to demonstrate that increase in cell volume corresponds to the zone of elongation (Chaly and Setterfield, 1975; Sunderland and Mcleish, 1961; Brown and Broadbent, 1950). An estimate of mean cell volume is usually obtained by dividing volume of the segment with the mean number of cells it contains, an estimate of which is commonly obtained by the maceration and cell counting technique of Brown and Rickless (1949). However, comparison of mean cell size is subject to similar objections to those for rate of cell expansion in that the apical millimetre consists of cells of widely divergent sizes and directions of growth. As mean cell size therefore provides no information on development of size of individual cells either from the apex of the root proper, or between different cell types in the meristem, it is more useful to analyse cell expansion from anatomical observations.

Cell length and volume alter throughout the apical 10mm. Again, as for cell radial and tangential diameters, cross-sectional

area (section 1 B i and ii) and cell division (section 1 b i), the pattern of development in cell length and total volume were found to depend on the tissue and area within the tissue. Contrary to the results obtained from zonal analysis, in which the meristem is regarded as a region of cells remaining constant in size and undergoing little or no expansion (Brown and Broadbent, 1950; Chaly and Setterfield, 1975), increase in both length and cell volume are very marked immediately beyond the quiescent centre (Fig.4:1:1, 4:1:5 and 4:1:11).

Procambial cells increase in length from 12μ at the quiescent centre junction to 35μ by 500μ from the quiescent centre (Fig. 4:1:1 and 4:1:11). The increase in cell length thereafter proceeds more slowly/unit length of root. Endodermal and pericycle cells also increase in length within the first 200μ from the quiescent centre, although to a lesser extent than procambial cells. The cortical cells however, do not increase in length within the apical mm, and may undergo a slight decrease in length. This corresponds to the observation of Jensen and Kavaljian (1958) for roots of *Allium cepa* where cortical cells have a period of constant length before elongating.

To a large extent the length of a cell in the meristem reflects its rate of transverse division relative to the rate of elongation of that zone. The actual rate of increase in cell length will therefore be obscured by the pattern of cell division in the apex, and will depend on the frequency and length of division and the fraction of cells cycling in different areas of the meristem in relation to the elongation rate of that zone. As mentioned in section 1 B i a, there do not appear to be areas or isolated cells that have ceased, or that are unusually active in, transverse division. It has also been found that although cells do vary in rate of division and length of the cell cycle (Barlow and MacDonald, 1973) that cells are continually displaced with respect to the apex and that all cells do appear to cycle, undergoing divisions according to their position within the apex and within any single group of cells derived from the same initial. In the case of cortical cells within any group of cells derived from the same initial, the divisions were found to be initially apical, becoming progressively more basipetal (Barlow, 1983). As cells are continually displaced by divisions

Figure 4:1:11. Developmental changes in cell length in a variety of tissues within the apical millimetre of pea root

Pea roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Median longitudinal sections were cut of the apical millimetre, and cell length in a variety of tissues measured directly with the aid of a micrometer eyepiece. Tissues measured include the procambium (—●—PCM), mid cortex (—▲—MC), outer cortex (—□—OC), inner cortex (—■—IC), endodermis (—▲—EN), pericycle (—△—P), epidermis (—●—EP), and inner root cap (—○—IRC).

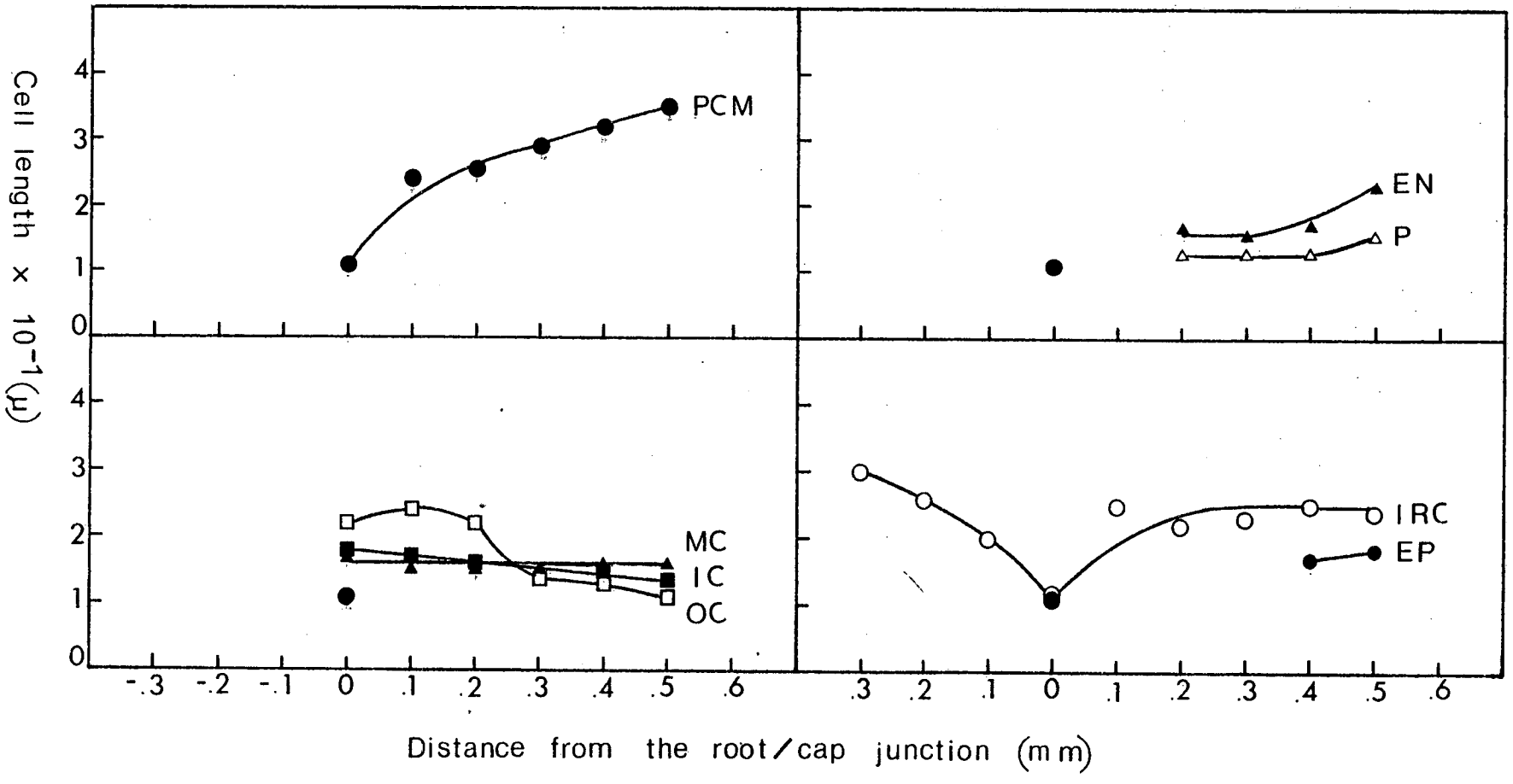

Cell length x $10^{-1}(\mu)$
PCM
EN
P
MC
IC
OC
IRC
EP
Distance from the root/cap junction (mm)

Fig. 4:1:11.

nearer the apex, cell division therefore continues throughout the meristem. It has already been noted that elongation rate/unit length of root varies within the meristem. However, it has been found (Erickson and Sax, 1956b; Goodwin and Avers, 1956) that the rate of cell proliferation/cell alters in a corresponding fashion. Rate of elongation/cell is therefore probably relatively constant throughout the meristem. Thus increase in cell length throughout the meristem reflects a growth rate constantly relatively greater than that required to compensate for cell division, rather than, for example, a relatively high rate of cell division at the quiescent centre boundary coupled with a low rate of elongation resulting in anomalously small cells at this zone, or a relatively low rate of cell division and high rate of elongation at the base of the meristem producing relatively large cells in this zone,

II. Cell volume

Cell volume as estimated from cell dimensions was also found to vary within the apical meristem. There was a considerable increase in cell volume within the first 500μ beyond the quiescent centre largely due to an increase in cell cross-sectional area (Fig.4:1:5a). This again contrasts with the results of Chaly and Setterfield who observed from mean cell values that there was very little increase in cell width in the root apex, increasing only 1.5-fold between 1 and 10mm from the apex. However, in these studies, within 500μ of the quiescent centre/root cap junction, metaxylem and mid-cortical cells underwent a 6.5-fold increase in volume; protoxylem and endodermis a four-fold increase; inner cortical cells a 3.5-fold increase; pericycle and outer cortical cells a 3-fold; and phloem cells a 2.5-fold increase in cell volume. The increase in cell volume/unit length of root declines thereafter.

It therefore appears that increase in cell volume more than compensates for cell division in the meristem. This is in contrast to the results of Chaly and Setterfield (1975) and Brown and Broadbent (1950) who claim from using mean cell volume in pea root that there is no increase (Chaly and Setterfield) or very little (Brown and Broadbent) increase in cell volume

within the meristem; the mean cell volume estimated by Brown and Broadbent to increase from $8.7cm^3 \times 10^{-9}$ at 0-0.4mm from the quiescent centre to $10.8cm^3 \times 10^{-9}$ at 0.4-0.8mm from the quiescent centre.

It therefore appears that rate of growth, rate of growth/cell and total growth/cell may be higher in the meristem than was indicated by measuring rate of elongation and measuring mean cell volume over relatively large areas. It also appears that elongation/cell does not correspond to expansion/cell within the meristem as increase in cell volume is largely due to increase in cell cross-sectional area. These values vary considerably between cells of different tissues from a very early stage in development, and it is interesting to note that although total expansion/cell will be different in different cells of the same transverse zone so that total increase in cellular material/cell will differ, that elongation rate/unit length of root over the meristem must be coordinated in different tissues. It was therefore of interest to investigate whether increase in any cell compartment followed any general trend in different tissues such as a greater increase in vacuome expansion than other compartments as implied by Brown *et al.* (1952) and Sunderland and McLeish, (1961) to occur during cell elongation. This was investigated at the ultrastructural level, and results will be described in section C i.

(iv) Summary

It appears that patterns of division, growth and differentiation in the pea root apex are far more complex than indicated by the model of Brown (1963, 1964). Both expansion and differentiation occur within the apical meristem, while division characteristics within the meristem appear to be dependent primarily on tissue type. Furthermore, division is not confined to cells of the apical meristem, but occurs also in relatively mature cells. The root apex therefore does not consist of longitudinal zones of qualitatively and inherently different capacities. The meristem is not a homogeneous mass of dividing cells which are incapable of growth and differentiation, and which are induced to differentiate at a late stage in the meristem.

Each tissue becomes apparent well within the apical meristem, and cells in each tissue follow a unique pattern of division, radial and tangential expansion, elongation and differentiation. This will be further discussed in section 1 E of this chapter, and the implications discussed in chapter 5.

(v) Recognition of tissues at the apex

As discussed in the previous sections, tissues may be identified at the apex largely by characteristics of cell shape and size, reflecting, in the apex and lateral root primordia, frequency and plane of division relative to the elongation and expansion rates of the zones. However, differential growth rates and polarity of growth were also found to contribute to changes in cell shape and size in older areas of the root. The different appearance of cells due to different cell division characteristics is perhaps, however, not an adequate criterion for judging differentiation as it might be argued that these characteristics are secondary to differentiation, or are independent of differentiation, being dependent on position within the apex and localised concentrations and ratios of cell division regulatory substances. Differences might arise directly from these conditions rather than from modified responses to cell division factors according to different capabilities to respond, or to programmed differences in division characteristics. This is particularly important from the developmental point of view as Brown (1963) has claimed that differentiation in the root does not and cannot commence until cells cease dividing and enter a growth phase beyond the meristem. Some tissues (for example, endodermis) are also frequently judged to be differentiated only after the cell divisions to produce the tissue are complete. In this case differentiation appears to be viewed as occurring after cessation of cell division; individual cells within these layers unable to commence differentiation without the presence of a full complement of cells.

I therefore examined ultrastructural features of development to see if ultrastructural differentiation occurred as near to the apex as structural differentiation, as well as to investigate ultrastructural characteristics of development.

Some of the developmental features observed are outlined in section 1 C of this chapter.

C. Ultrastructural development of tissues during differentiation

The ultrastructural development of cells at late stages of maturation has been well documented, in particular the development of vascular tissues, root cap and epidermis. Studies of the early stages of differentiation are much more rare, as are studies following the differentiation from newly initiated to the fully mature state. In sections C and D I have therefore outlined some of the ultrastructural characteristics of cells when they are in initial stages of differentiation and which may be used to distinguish them from adjacent cells. The features of development of one organelle, the vacuole, were followed in different tissues, and are described in section C i. Other ultrastructural features will be described where appropriate.

(i) Vacuole development

At the level of the light microscope, tissues may be recognised largely by cell shape and size, by staining properties, and by the degree and timing of vacuolation of their cells. I therefore investigated the ultrastructural development of vacuoles throughout the apical 20mm of pea root. Striking differences in vacuole development were found between different tissues.

a) Vacuole formation

I. Vacuole ontogeny

In the quiescent centre and apical 100μ of the promeristem, terminal and intercalated dilations of the R.E.R. were frequently observed (Fig.4:1:12). Rough E.R. protrusions from the nuclear envelope also frequently dilate terminally, and dilations direct from the outer nuclear envelope may also be seen (Fig.4:1:12). These regions usually contain some electron-dense fibrillar material similar to that found in vacuoles and provacuoles of the

Figure 4:1:12. Vacuolar ontogeny

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation under the electron microscope. Vacuolar ontogeny was examined from longitudinal sections. Micrographs shown are at 1) 10μ, and at b) - d) 100μ from the apex of the root proper in cortical tissue.

Provacuoles (pv) may arise *de novo* by terminal or intercalated dilation of rough endoplasmic reticulum (drer). Provacuoles may also be observed to be continuous with the outer nuclear membrane (onm).

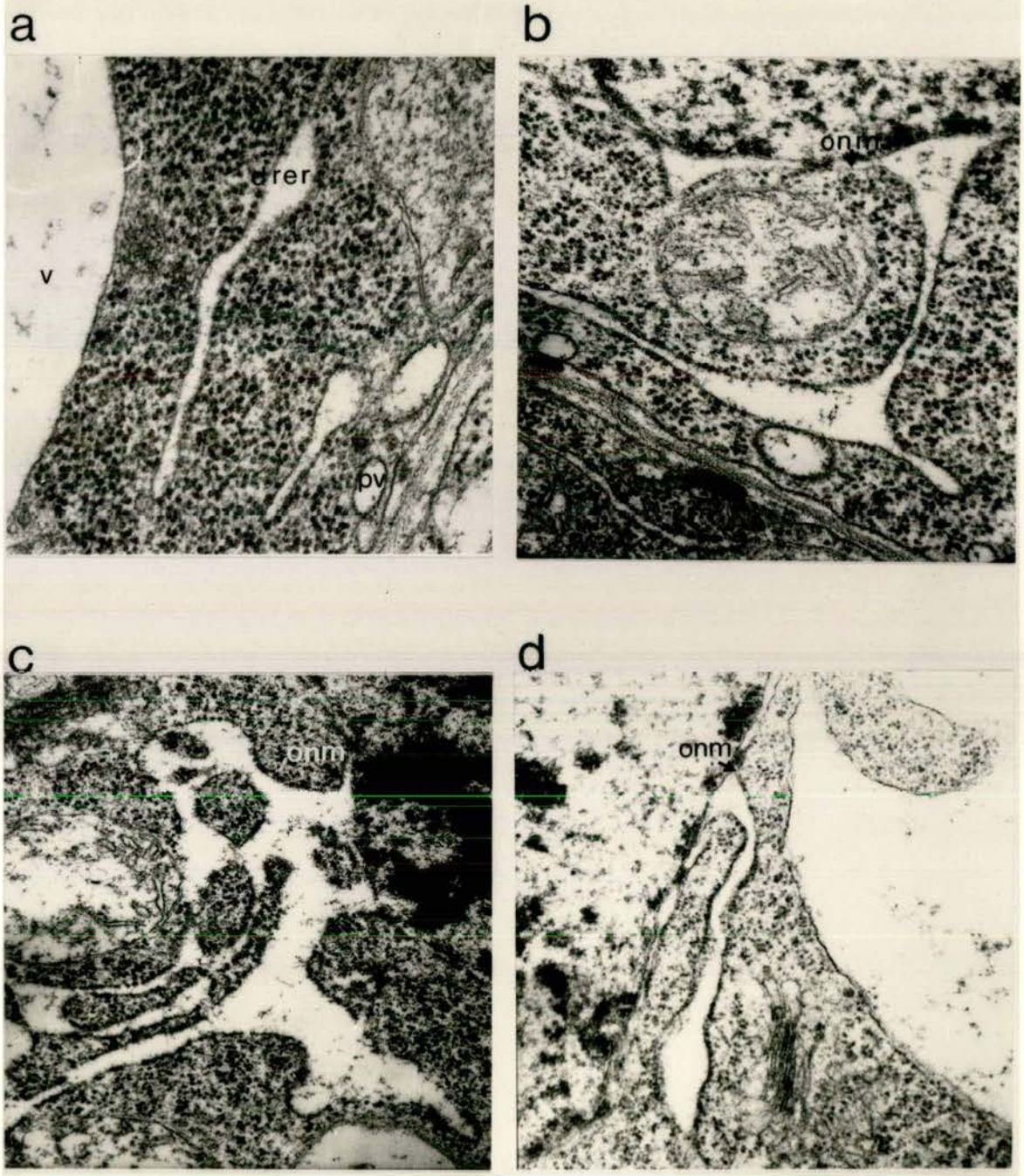
a) x 55,200

b) x 41,400

c) x 27,600

d) x 35,100

Fig. 4:1:12.



apex (Fig.4:1:12 amd 4:1:13). As many narrow filaments of R.E.R. and Golgi bodies are also observed (Fig.4:1:12b and d) and there is no evidence for poor fixation of other components of the cells the dilated regions are probably not artifacts of fixation. These observations support the proposition that vacuoles may arise *de novo* from R.E.R. and from the outer nuclear envelope in pea root, as has been observed in other plant root cells (Mesquita, 1969; Poux, 1962) and which includes evidence obtained from freeze-etched sections (Matile and Moor, 1968), enzymatic studies of the E.R. and tonoplast (Matile, 1968), and comparison of the fine structure of E.R. and tonoplast membranes in freeze-etched sections (Matile and Moore, 1968). No evidence for origin from Golgi bodies (Matile, 1975), from local cytoplasmic hydration, or by fission of existing vacuoles was obtained in these studies of the pea root.

II. Vacuole expansion

Vacuole expansion appeared intially to occur in most cells by a combination of fusion and inflation as well as by expansion of existing vacuoles. In the cortex, many irregularly-shaped provacuoles had formed from R.E.R. by 100μ from the root cap boundary. These provacuoles became more rounded and enlarged as they progressed from the tip (Fig.4:1:13a) indicating that inflation was occurring more rapidly than increase in area of the tonoplast.

Enlargement also appeared to occur by fusion: individual vacuoles were observed to protrude into another vacuole (Fig. 4:1:13a and b), the tonoplasts presumably fused, and the segments of tonoplast isolated from the cytoplasm broke down (Fig. 4:1:13c and d) to form a single vacuole which might undergo further expansion and fusion (Fig.4:1:13d). As this sequence has also been recorded in freeze-etched root tip cells (Matile and Moor, 1968) it is unlikely to be an artifact of chemical fixation. It is also unlikely that the stages represent fission, as the sequence proceeded from a large number of small vacuoles to a small number of larger vacuoles as the cells aged. Furthermore, the contents of two vacuoles appearing to fuse may differ (Fig.4:1:13b).

Figure 4:1:13. Vacuolar expansion

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation by the electron microscope. Electron micrographs are of transverse sections of cortical tissue at a) - c) 100μ, and d) 300μ from root apex.

Vacuolar expansion may occur by fusion and/or inflation. Vacuolar fusion is preceded by the protrusion (pr) of one vacuole into another (figures a and b). The tonoplasts appear to fuse (f), the isolated membrane then breaks down, and vacuolar contents mix. Several vacuoles may fuse into one larger vacuole (v) (figure d).

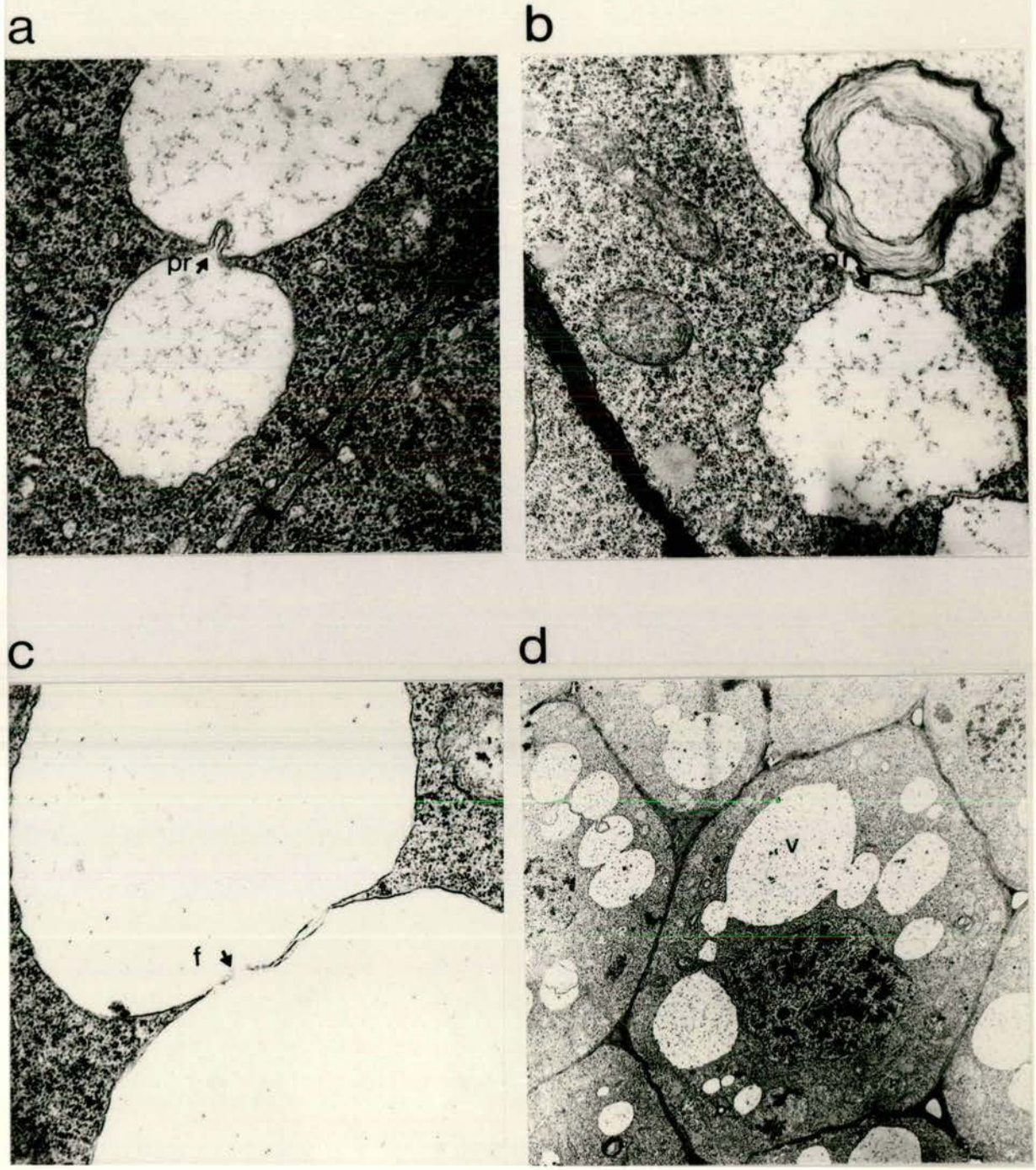
a) x 27,600

b) x 18,700

c) x 18,000

d) x 4,700

Fig. 4:1:13.



III. Vacuolar ontogeny and expansion in different cell types

By 200μ from the root cap/quiescent centre junction vacuoles have developed from R.E.R. in cortical cells and have undergone extensive fusion and inflation, although fusion continues throughout the apical 2mm. Rough E.R. dilation was not observed in procambial, protoxylem, metaxylem or protophloem cells. However, provacuoles in metaxylem cells undergo rapid fusion and inflation within 100μ of the quiescent centre/cap junction and enlarge greatly to become the largest vacuoles by 200μ. In protoxylem cells only a few small vacuoles were observed until later fusion and inflation. It therefore seems probable that a small number of provacuoles are inherited at division from quiescent centre cells which do undergo vacuolar ontogeny and presumably therefore increase the number of provacuoles/cell continually. Rough E.R. dilation and vacuole formation were observed in metaphloem cells, but this did not commence until 1.5-2mm from the tip, after which fusion of provacuoles was observed up to about 4mm from the tip. Little fusion and inflation occurred in these cells, however, and vacuoles remained relatively small before undergoing deflation during differentiation. Protophloem sieve elements contained very few vacuoles early in differentiation, possibly being derived from quiescent centre cells. Unlike the vacuoles of metaphloem sieve elements these do not appear to fuse, and undergo little expansion, as observed in differentiating sieve elements of other species (Esau and Gill, 1972; Bouck and Cronshaw, 1965).

Thus, the low vacuome fraction of phloem cells as observed by the light microscope in the apical 2mm (Fig.4:1:4d) appears to be due to late vacuolar ontogeny with a low rate of ontogeny in metaphloem sieve elements; and absence of vacuolar ontogeny, with low rate of inflation and fusion of provacuoles inherited from the quiescent centre cells in protophloem sieve elements. By contrast, the presence of a small number of large vacuoles in xylem cells in the apical 2mm as observed by the light microscope (Fig.4:1:1 and 4:1:4d and e) appears to be due to a low degree of vacuolar ontogeny coupled with rapid early fusion and inflation of provacuoles inherited from quiescent centre cells. The large number of small vacuoles observed in the cortical cells

appear to arise by a high degree of provacuole ontogeny, and a relatively low rate of fusion and enlargement.

Thus, vacuolar ontogeny, fusion, inflation and enlargement were all found to vary between different cell types in the rate at which, and the extent to which they occurred, and in the distance from the quiescent centre at which they occurred.

b) Vacuole development during cell differentiation and maturation

All cell types have unique characteristics of vacuole development during differentiation. These are described below.

I. Cortex, epidermis, endodermis and pericycle

After extensive vacuolar fusion has taken place, and the vacuome fraction has reached about 1/3 of the total cell volume, extensive invaginations of the tonoplast commence (Fig.4:1:14a-c). The invaginations, which may include organelles, appear to be 'pinched off', and become free in the vacuolar sap as membrane-bound particles which are generally round or oval in shape (Fig.4:1:14b-d). As the vacuole expands, these inclusions at first rise in number, but then decline both in number and in the electron-density of their contents, and appear to be in the process of internal degeneration (Fig.4:1:14d). As they are not observed in highly vacuolate cells where the vacuome occupies more than 2/3-3/4 of the cell volume, presumably the membrane eventually breaks down (Fig.4:1:14e). Degeneration probably occurs as a result of prolonged exposure to hydrolytic enzymes and isolation from the nucleus and the rest of the cytoplasm which may prevent continued replacement of tonoplast constituents.

In these cells, autophagy occurs when the vacuome occupies one- to two-thirds of the cell volume. It is therefore first observed in mid-cortical cells as these undergo earlier and more rapid vacuolar expansion than adjacent cells. Autophagy is most active in the root 2-4mm from the tip, although it continues to be observed for the entire 20mm in endodermal, pericycle and inner cortical cells adjacent to the protoxylem arcs which remain meristematic and have a higher cytoplasm/

Figure 4:1:14. Vacuole development in cells of the cortex, epidermis, endodermis and pericycle

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation under the electron microscope.

Electron micrographs are of

a) - c) the inner cortex, 3mm from the root apex

d) the inner cortex 20mm from the root apex

d) the mid cortex 3mm from the root apex

Autophagy commences in cells of the cortex, epidermis, endodermis and pericycle when the vacuole occupies approximately $\frac{1}{3}$ of the cell volume, and continues until the vacuole occupies more than approximately two thirds of the cell volume. Autophagy occurs by invagination (i) of the tonoplast, followed by 'pinching off' of these invaginations and release into the vacuolar sap as membrane bound particles (mbp). As the vacuole expands, frequency of invagination declines, and the number of membrane bound particles increases and the proportion of these particles apparently undergoing internal degeneration (dmbp) increases. In highly vacuolate cells, membrane bound particles are very rare, presumably as the surrounding membrane eventually breaks down and is autolysed.

Electron-dense amorphous bodies (edab) are also commonly observed in autophagic cells either in association with the tonoplast or in a large mass usually associated with membrane bound particles.

a) x 8,300

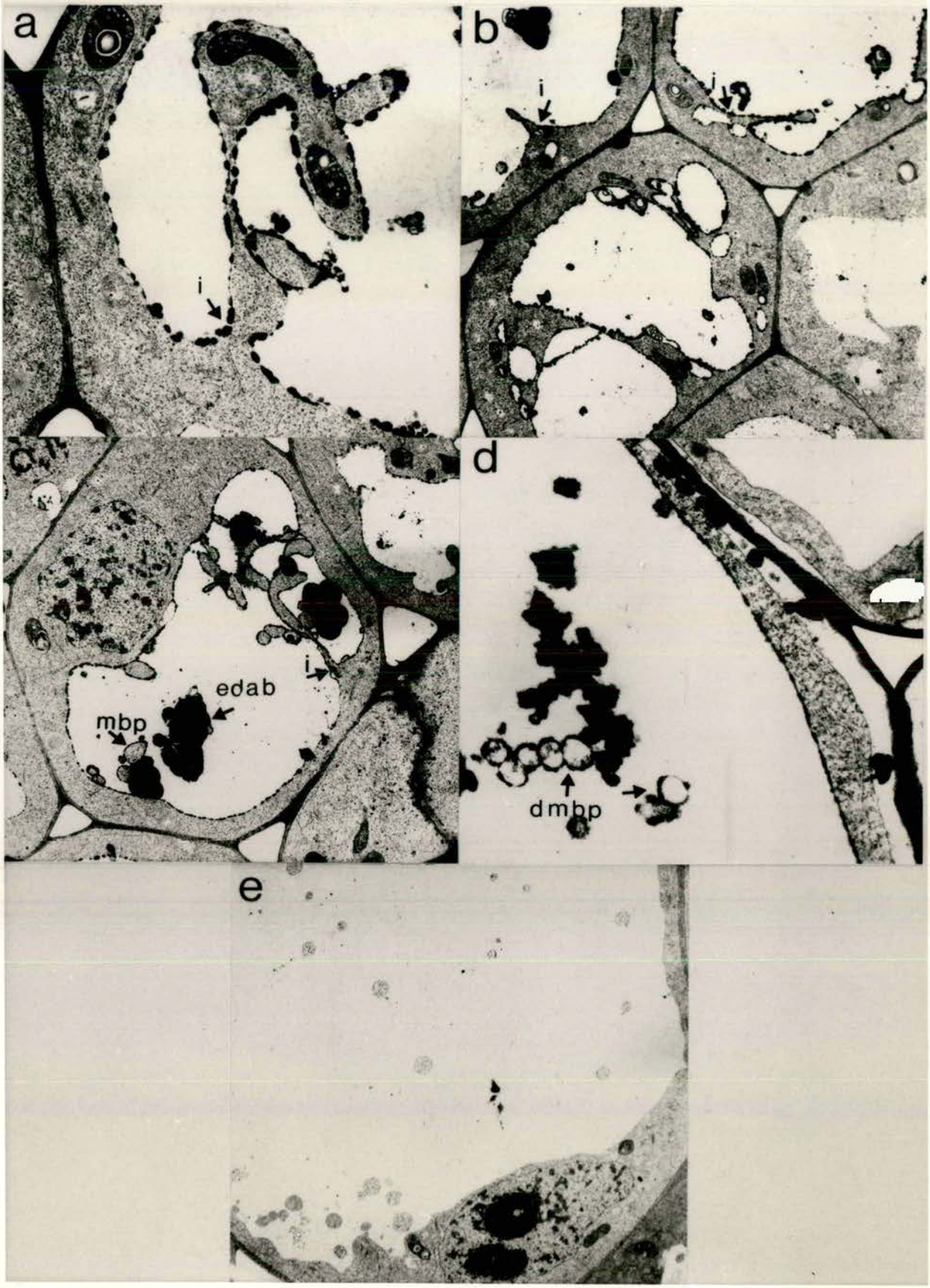
b) x 3,500

c) x 3,500

d) x 11,700

e) x 2,100

Fig. 4:1:14.



vacuome ratio than their neighbours.

Autophagy has been recorded in a number of cells, including root tip cells using freeze-fracturing for the scanning electron microscope, and serial sectioning for the transmission electron microscope (Fineran, 1971; Esau, 1974; Matile and Moor, 1968; Matile, 1975), and it appears that the fine structure of the tonoplast is different at the site of invagination as observed in freeze-etched cells (Fineran, 1970a), indicating that it may be an active process.

Highly electron-dense amorphous inclusions are observed at about the same time as autophagy in these cells, appearing first in the outer cortex and being observed progressively nearer to the stele as the vacuome expands. The inclusions are present either free in the vacuolar sap, or more usually as globules in association either with the tonoplast or membranes of cytoplasmic inclusions (which were originally part of the tonoplast) (Fig. 4:1:14). By 2mm from the tip they are prominent in the inner cortex, pericycle and endodermis. The material eventually breaks down, becoming fibrous, and disperses throughout the vacuolar sap (Fig. 4:1:14e), although remaining more prominent in autophagic vacuoles of the lateral root primordia. It is possible that this material is derived from breakdown of the cytoplasmic inclusions. This is supported by the observation that the only other cell type in which they are observed is the metaphloem sieve element during the brief period of autophagy in these cells. Autophagy is not observed in any other type of cell.

II. Root cap

Immediately distal to the quiescent centre, the root cap cells of the middle columella contain a few small vacuoles (Fig. 4.1.15a). As cells progress towards the tip and enlarge, the vacuoles become smaller, reduced in size and angular, indicating that deflation may have occurred. Although chemical fixation may alter vacuolar morphology (O'Brien *et al.* 1973) there was no evidence of poor fixation in the root tip, while vacuolar deflation of root cap vacuoles did not resemble that caused by poor fixation (Fineran, 1970b). Deflation is therefore unlikely to be an artifact of fixation. The vacuolar sap also becomes

Figure 4:1:15. Vacuole development in cells of the root cap

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation under the electron microscope. Electron micrographs are of longitudinal sections of the root cap.

Vacuoles (v) in cells of the root cap columella adjacent to the root proper tend to be small and relatively rounded as seen in figure a. As cells age and progress towards the tip, the vacuoles become irregular in shape and rather smaller (figure b) indicating they may have deflated (dv) slightly. They concurrently become filled with highly electron-dense material. Plastids (P) tend by this stage to be large, highly electron-dense, and to contain large starch grains (St). Golgi bodies are also abundant. As cells progress further towards the periphery of the root cap the vacuoles enlarge and become more rounded (figure c), while starch grains in the plastids tend to become smaller and less abundant. The electron density of the vacuolar sap decreases as vacuoles continue to enlarge (figure d), and eventually the tonoplast breaks down and rapid cytoplasmic autolysis occurs.

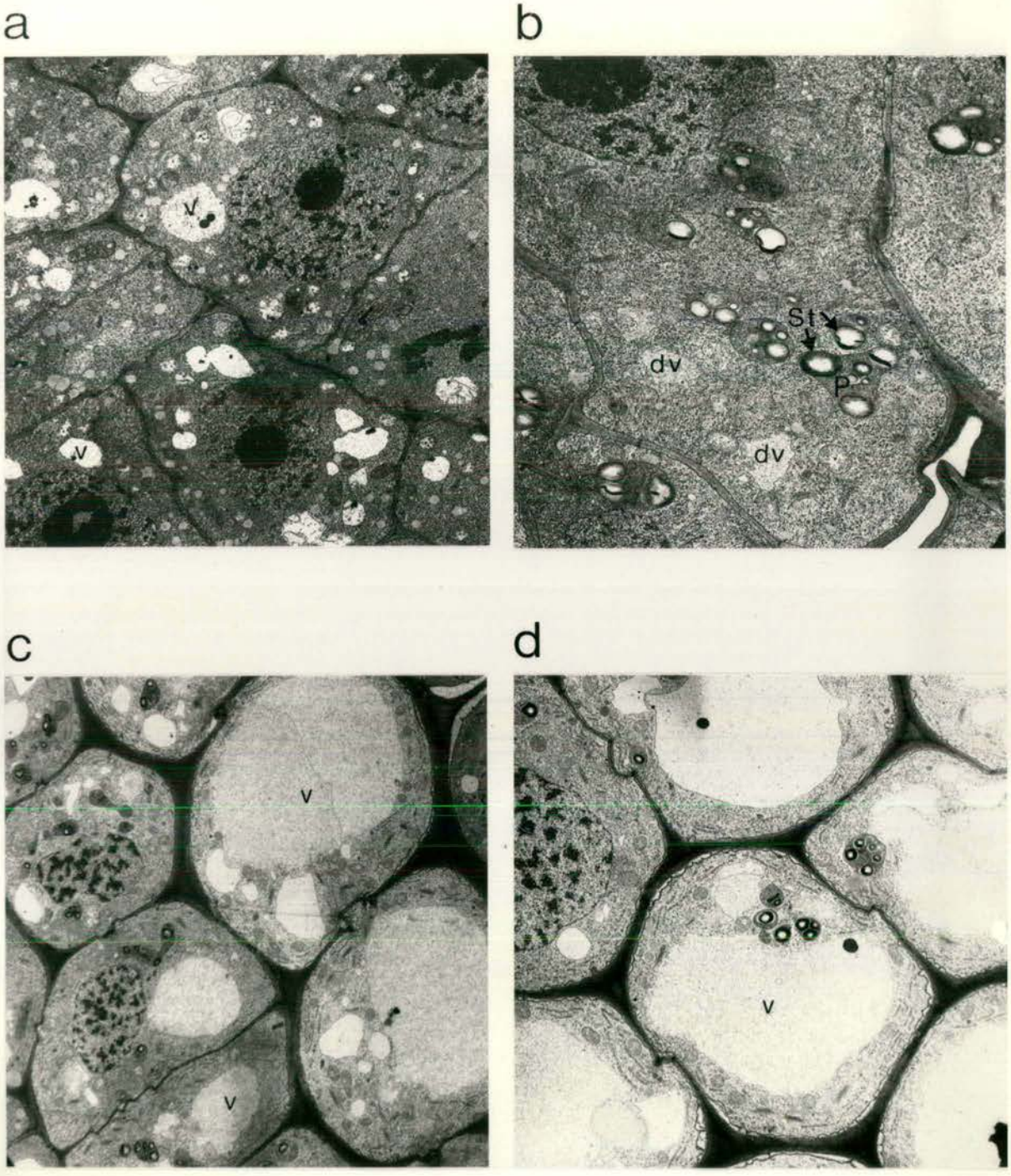
a) x 3,450

b) x 5,520

c) x 2,070

d) x 4,680

Fig. 4:1:15.



filled with finely granular electron-dense material (Fig. 4.1.15b). As cells are further displaced towards the periphery of the root cap and enlarge further, vacuoles begin to increase in size, become much more rounded and enlarged (Fig.4:1:15c) while the electron-density of the sap declines (Fig.4:1:15d). Eventually the tonoplast breaks down and rapid autolysis occurs.

The progression from an irregularly-shaped vacuole with highly electron-dense sap during early differentiation to a large vacuole with sap of low electron-density has been noted in root cap cells of other species (Fineran, 1966). It seems possible that this is related to production and distribution of osmotically active solutes as it is known that (^{3}H) glucose in young root cap cells is mainly incorporated into starch (Northcote and Pickett-Heaps, 1966) and is stored in plastids, whereas in the peripheral cells there is a far higher rate of (^{3}H) glucose incorporation (Barlow, 1974) most being utilised in polysaccharide slime production (Northcote and Pickett-Heaps, 1966). Thus sugars may be converted to starch in young root cap cells (with deflated vacuoles), but mobilised to increase available sugars for slime synthesis in older cells thus increasing the availability of osmotically-active material and allowing vacuolar expansion.

III Xylem

Xylem cells tend to form large central vacuoles which expand until secondary wall formation and lignification are well established (Fig.4.1.16a) as recorded in other plant species (Cronshaw, 1965; Cronshaw and Wardrop, 1964; Charvat and Esau, 1975; Wooding and Northcote, 1964). The tonoplast then ruptures and rapid cytoplasmic degeneration follows (Fig. 4:1:16b) again as described for xylem in other species (Cronshaw and Bouck, 1965; Wodzicki and Brown, 1973; Esau and Charvat, 1978). Mitochondria, plastids and membranes tend to be the last to break down (Fig.4:1:16b). Eventually no cell contents are visible (Fig.4:1:16c).

IV Phloem

Phloem tissue in pea root was found to consist largely of

Figure 4:1:16. Vacuole development in xylem cells

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation under the electron microscope. Electron micrographs are of xylem tissue obtained at a) 15mm and b), c) 20mm from the root apex.

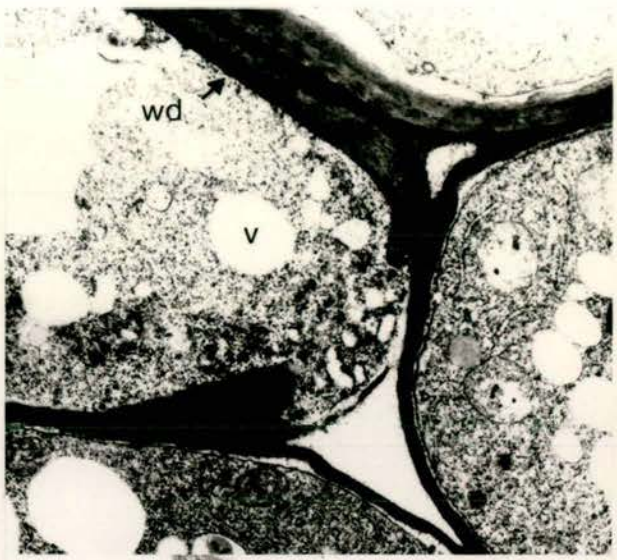
Vacuoles of xylem cells tend to enlarge and remain intact until a late stage of secondary wall deposition (wd) (figure a). The tonoplast then ruptures and rapid cytoplasmic autolysis follows. Mitochondria (m), plastids (p) and membranes (me) tend to be the last to degenerate (figure b). Eventually no cell contents remain (figure c).

a) x 15,200

b) x 9,400

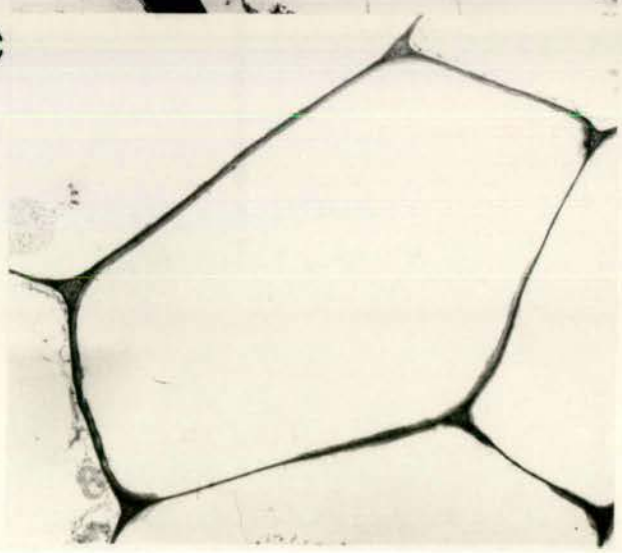
c) x 4,700

Fig.4:1:16. a



b

c



protophloem and metaphloem sieve elements, and sclerenchyma. Sieve elements appeared to undergo different developmental sequences depending on whether they were derived from protophloem or metaphloem.

Protophloem sieve elements

During secondary wall formation in sieve element differentiation, only a few small vacuoles are observed. Later in differentiation, as the cytoplasm becomes less dense and the nucleus begins to break down, the vacuoles enlarge slightly but do not appear to fuse to any great extent (Fig.4:1:17a). Breakdown of the tonoplast or rapid deflation of the vacuoles to the size of E.R. cisternae then occurs, followed by rapid degeneration of most of the cytoplasm (Fig.4:1:17b) with only mitochondria, plastids, and membranes including the sieve tube reticulum, being retained (Fig.4:1:17c and d). Sieve elements may eventually become crushed by adjacent cells (Fig.4:1:17d).

Metaphloem sieve elements

Differentiation of metaphloem sieve elements differs from that of protophloem sieve elements in several respects. These cells become densely protoplasmic by 2mm from the root apex, and appear to have a very low vacuome content (Fig.4:1:18a). After development of secondary wall structure (less than in protophloem sieve elements) at about 1.5-3mm from the tip, vacuoles arise within the highly electron-dense cytoplasmic matrix by dilation of the rough E.R. and outer nuclear membrane (Fig.4:1:18b). During a brief period of autophagy (which may exclude mitochondria and plastids as these were not observed in any tonoplast invaginations or vacuolar inclusions (Fig.4:1:18b)), the vacuoles enlarge slightly and the vacuolar sap becomes more electron-dense. Soon afterwards the vacuoles deflate and become irregular in outline concurrently with a more rapid reduction in electron-density of the cytoplasm, largely due to a reduction in ribosomes so that E.R. becomes more prominent (Fig.4:1:18c). Degeneration of the cytoplasm and nucleus proceeds until very little ground plasm remains (Fig.4:1:18d and e). Eventually, as in protophloem sieve elements, only mitochondria plastids and membranes (possibly

Figure 4:1:17. Vacuole development in protophloem sieve elements

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation by the electron microscope. Electron micrographs are of protophloem sieve elements

a) 400µ from the root apex in longitudinal section. Mag. x 3,500

b) 3mm from the apex in transverse section. Mag. x 8,300

c) 3mm from the apex in transverse section. Mag. x 2,100

d) 5mm from the apex in transverse section. Mag. x 8,300

Vacuoles (v) in protophloem sieve elements are initially small, and the tonoplast remains intact while the secondary wall develops (wd) and initial stages of nuclear (N) and cytoplasmic breakdown occur. As cell breakdown progresses, the sieve tube reticulum (STR) develops and appears to be aligned in parallel stacks along the walls. Mitochondria (m), plastids (p). membranes (me), and the sieve tube reticulum remain in the cell at maturation.

Fig. 4:1:17.

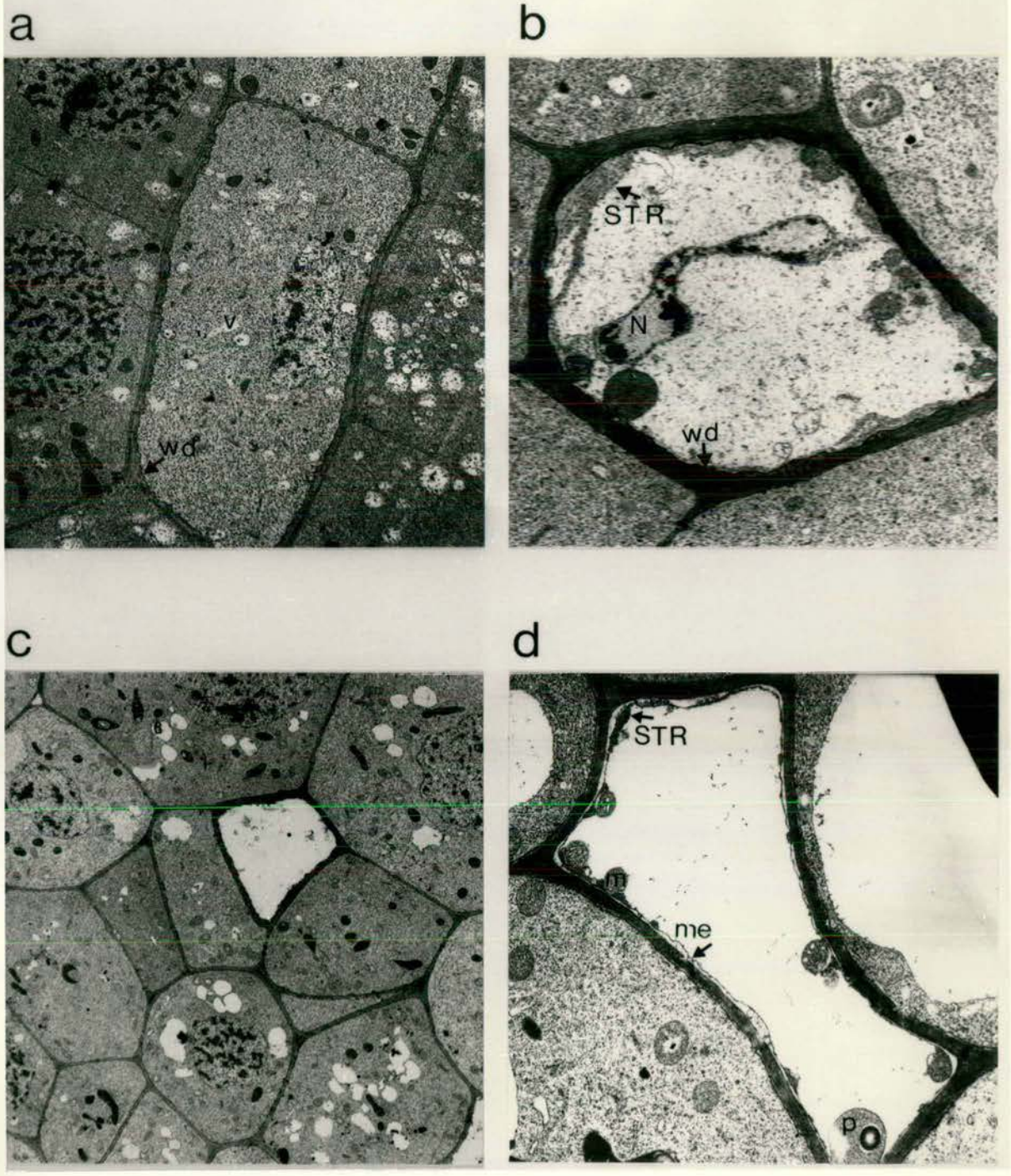


Figure 4:1:18. Vacuole development in metaphloem sieve elements

Pea roots were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite as described in chapter 2 section 2 D. Sections were stained with Reynolds' lead citrate for observation under the electron microscope.

Electron micrographs are of metaphloem sieve elements a) - d) 2-3mm, e) 6mm, and f) 4mm from the root apex.

Provacuoles (pv) appear to arise *de novo* from dilations of rough endoplasmic reticulum (drer) at a relatively late stage of cell development. A brief period of autophagy (a) is then associated with the newly formed vacuoles (v). The cytoplasm then begins to degenerate, vacuoles deflate (dv), and it is possible that the tonoplast breaks down before cytoplasmic degeneration is complete. Eventually mitochondria (m), plastids (p), and membranes (me) remain in the cell at maturation.

a) x 13,800

b) x 8,300

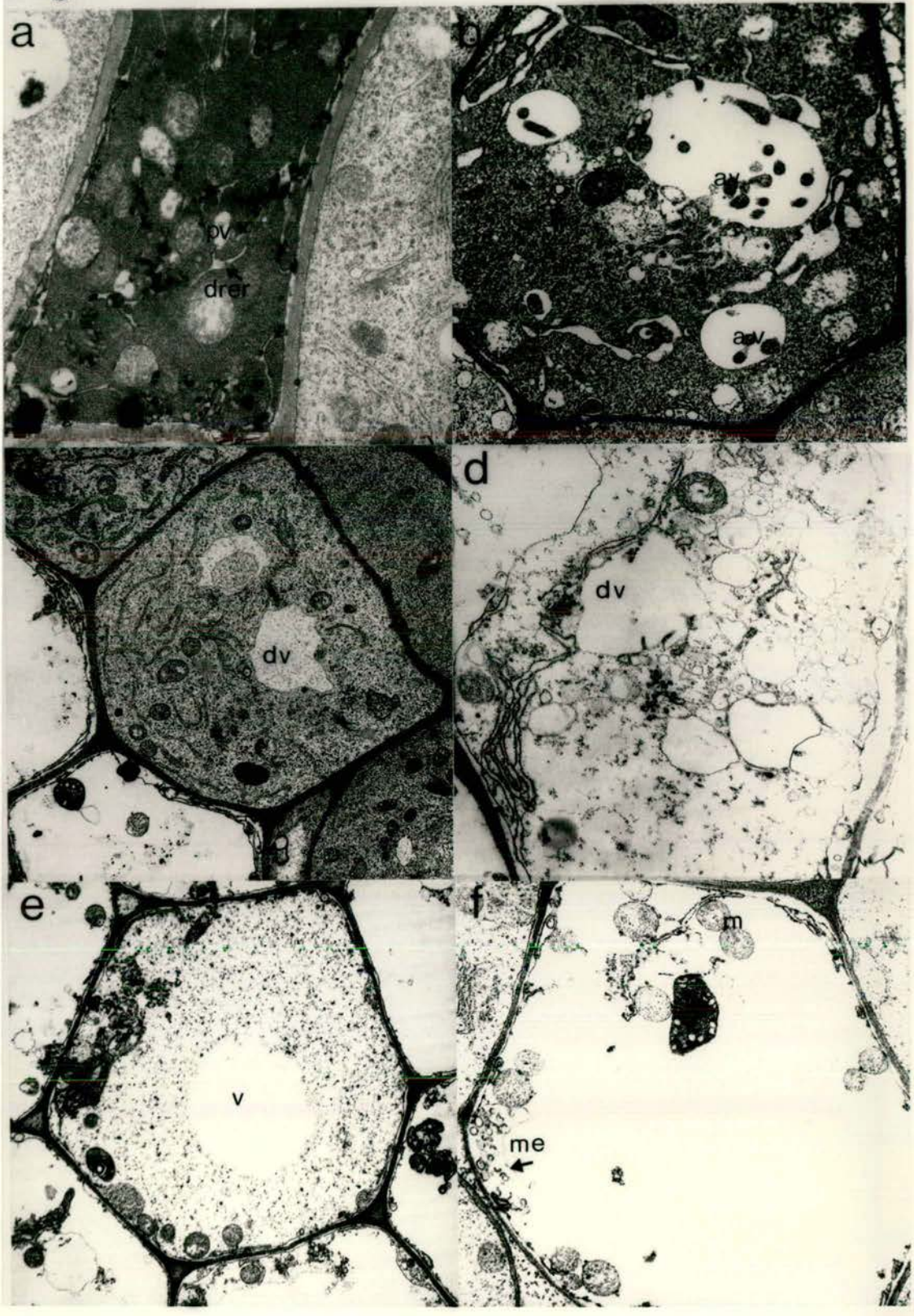
c) x 5,500

d) x 11,700

e) x 5,500

f) x 8,300

Fig. 4:1:18.



including deflated vacuoles) remain (Fig.4:1:18f). The inner metaphloem cells are the first to mature, shortly after 2mm from the tip. By 5-6mm most of the outer metaphloem cells have also matured.

It is not clear whether the tonoplast breaks down during differentiation of sieve elements (Fig.4:1:18e), or whether the vacuoles deflate (Fig.4:1:18d) to the size of E.R. filaments thus maintaining the lytic compartment at least partially intact as appears to occur in sieve elements in some species (Heyser, 1971). In the case of pea root, it is possible that differences occur between metaphloem and protophloem sieve elements. Metaphloem appears to undergo obvious deflation of the vacuole which may well deflate to the size of E.R. without breakdown of the tonoplast. The vacuoles of protophloem sieve elements, however, disappear suddenly, and it seems more likely that the tonoplast breaks down. There is some controversy as to whether breakdown of the tonoplast occurs or not in other species (Esau and Gill, 1972; Bouck and Cronshaw, 1965; Heyser, 1971) and it is possible that this reflects differences between protophloem and metaphloem differentiation.

It is also interesting to note that vacuolar autophagy in sieve element differentiation has been observed by some authors (Buvat, 1968) whereas no mention of the phenomenon is made by others (Esau and Gill, 1972). As autophagy was observed in metaphloem but not in protophloem of pea root, again it is possible that this discrepancy may be a result of different developmental sequences in the two tissues.

c) Summary

Vacuole development, as well as vacuole ontogeny and expansion, has features unique to different cell types. The most striking features of vacuole development are the apparently random autophagy in cortical, epidermal, pericycle and endodermal cells; maintenance of the tonoplast until a late state of maturation in root cap and xylem cells, with rapid autolysis following tonoplast rupture; breakdown of the tonoplast or rapid deflation of the vacuole followed by selective degeneration in protophloem

sieve elements; selective autophagy followed by vacuole deflation concurrent with selective cytoplasmic degeneration in metaphloem sieve elements.

Differences in vacuole development observed at the light microscope level may therefore be correlated with differences in both vacuole formation and vacuole development, and these differences may therefore be used to identify cells undergoing differentiation.

D. The order and level of tissue differentiation in the root apex

The order and level of tissue differentiation and maturation have been obtained for roots of several species. It is generally found that the order remains the same irrespective of growth conditions, although the distance from the apex at which differentiation and maturation occur do depend on treatment. Values in this section therefore refer to roots germinated for 65 hours at 22°C that were 2.5-3.5cm long.

(i) Criteria used to judge the level of differentiation

Differentiation in this context has been rather arbitrarily and inconsistently defined, the definitions appearing to vary with the tissues concerned. For example, the endodermis is generally judged to be 'differentiated' when periclinal and frequent anticlinal divisions no longer appear in the inner cylinder of cortical cells. Similar criteria are used to judge differentiation of pericycle (for example, Popham, 1955). Metaxylem, on the other hand, is generally judged to be differentiated on the basis of early cessation of cell division of central stelar cells allowing rapid cell enlargement, and protoxylem on the basis of differential enlargement and vacuolation from other cells in the stele (Peterson, 1967; Popham, 1955) or of secondary wall formation. Phloem differentiation is judged to commence when one cell of one specialised cell type, the sieve element, first appears (Heimsch, 1951; Popham, 1955; Esau, 1941).

The criteria for judging a tissue to be differentiated are

therefore inconsistent. Using the criterion applied to endodermal differentiation, tissues might be judged to be differentiated when their cell lineage had a full complement of cells. Thus differentiation could be declared irrespective of whether any structural or molecular differentiation had taken place, differentiation being assumed on the basis that these cells were in the same lineage as older differentiating cells. Also by this definition cells would be viewed as not being in the process of differentiation until the entire layer of cells had ceased dividing, even if many cells already had developed characteristics of that tissue. Perhaps it was this consideration that prompted Brown (1963) to claim that differentiation does not take place until cell division ceases in the root apex.

However, differences in structural and ultrastructural development between cells may be clearly detected even within the quiescent centre indicating that differentiation is occurring in all cells long before they cease dividing, and the criterion of a full (or nearly full) complement of cells is therefore perhaps too late a stage in development to use as an indicator of differentiation, as existing cells within the zone will have already initiated differentiation and may have spent a considerable time in the process of differentiation. Furthermore, in the case of the endodermis in pea root, I have observed that divisions in the inner cortex and endodermis are common 200-300μ from the quiescent centre, and continue throughout the apical millimetre, whereas Popham (1955) describes these cells as the first cells to differentiate in the pea root apex on the basis of early cessation of cell division. Similarly, the criterion of differentiation of one specialised cell type to judge differentiation of phloem cells is perhaps inappropriate as phloem consists of several cell types while the pattern of the 'unspecialised' protophloem and metaphloem cells may be clearly discerned by 100-250μ from the quiescent centre at both the structural and ultrastructural levels (Fig.4:1:4c and d). The first sieve element however, is not observed for a further 100μ beyond this and in a sense represents secondary differentiation. Similarly, formation of secondary thickening in xylem walls which is sometimes used to judge differentiation, is in fact a late stage of

maturation, and does not apply to the initial formation of a different cell type.

As differentiation may be viewed as any programmed developmental change that causes cells to diverge in molecular or structural character, I have therefore chosen a more nebulous criterion for judging differentiation at the cytological level, judging it to have occurred when most of the cells in a layer or zone acquire distinctive and individual cytological characteristics of that zone to the extent that they may be clearly identified as belonging to a particular cell type. The characteristics used in identifying cell types include cell shape and size, vacuole formation and development, plastid and Golgi body development, and starch and lipid content. It should be noted that differentiation of individual cells may be observed prior to the zone at which differentiation is judged, and that differentiation must probably have proceeded for some time to allow cells to diverge sufficiently cytologically to be identified as different. Some of the characteristics of tissues as they initially emerge are briefly outlined in the following section.

(ii) The order of tissue differentiation and characteristics of tissues at early stages of differentiation

The order of tissue differentiation is shown in table 4:1:1.

Distal to the quiescent centre at the top of the root cap columella root cap cells are immediately identifiable by their irregular walls, large cell volume containing weakly staining cytoplasm, few small vacuoles, and large starch grains (fig.4:1:1). Rough E.R. dilation and provacuole formation were not observed in these cells. Plastids, Golgi bodies, mitochondria, lipid and starch grains are all abundant (Fig.4:1:15); and plastids, which have a much more electron-dense matrix than those in the adjacent quiescent centre or cortex, enlarge and may develop rapidly into amyloplasts containing large starch grains (Fig. 4:1:15b).

Cortical cells can be discerned immediately adjacent to both procambial cells within the quiescent centre and the root cap (figures 4:1:1 and 4:1:19a). These cells have a smaller cross-sectional area than root cap cells, but have up to three

Figure 4:1:19. The apical meristem of *Pisum sativum*

Roots were prepared for observation under the light microscope as described in chapter 2 section 2 C ii. Transverse sections were cut at a) 10μ, b) 50μ, and c), d) 300μ from the apex of the root proper.

Procortical cells (PCX) may be discerned at a very early stage from procambial cells (PCM) by their large cross-sectional area, higher degree of vacuolation, larger vacuoles and less densely staining cytoplasm A large number of more specialised cell types including epidermis (EP), endodermis (EN), pericycle (P), protoxylem (PX), metaxylem (MX), phloem (PH), and sieve elements (SE) are clearly distinguishable within the apical meristem by a variety of characteristics as described in figure 4:1:4, and in section 1 B v.

a) x 300

b) x 300

c) x 300

d) x 300

times the cross-sectional area of procambial cells depending on the position within the cortex. They have a relatively weakly staining cytoplasm with a large number of small vacuoles. Extensive vacuolar ontogeny from rough E.R. and the outer nuclear membrane may be observed;and vacuolar fusion and inflation commences with 100μ of the root cap boundary. Plastids have a more electron-dense matrix and contain more starch grains than those in the procambial cells within the quiescent centre.

Procambial cells are identifiable immediately proximal to the quiescent centre/root cap junction due to rapid elongation, small cross-sectional area, dense cytoplasmic staining, and low rate of vacuome development with no detectable rough E.R. dilation (Fig.4:1:1, 4:1:4 and 4:1:19b). By 75-100μ from the root cap columella several cell types are becoming discernable within the procambium (Fig.4:1:4b). Metaxylem is distinguishable early in the procambial cylinder by more rapid expansion in cross-sectional area and vacuole fusion and inflation than cells of the outer stele (Fig.4:1:4). The vascular cambium is also beginning to delimit the inner boundary of the phloem arcs (Fig.4:1:4a and b). Endodermal and pericycle cells are also beginning to emerge visually at this stage, tending to be tangentially elongated (Fig.4:1:4a and b). They are less vacuolated (as observed in *Azolla* roots (Barlow *et al.* 1982)), have a lower lipid and starch content than the cortex, more highly electron-dense plastids, and a higher nuclear/cytoplasmic ratio. Endodermal cells differ from pericycle cells initially by a lower radial/tangential ratio (Fig.4:1:7a) due to shorter radial walls and longer tangential walls (Fig.4:1:8a and 4:1:9a) and by a much lower lipid content. They also differ by commencing fusion of vacuoles earlier than pericycle cells thus producing fewer but larger vacuoles.

By 200μ from the quiescent centre/root cap junction the triarch vascular pattern is becoming well established (Fig.4:1:4c) and epidermal cells (Fig.4:1:19d) are identifiable by a very high radial/tangential ratio, extremely well-developed mitochondria and highly electron-dense cytoplasm. Plastids are less electron-dense than those of the outer cortex, as observed by Whatley and Gunning (1981) for *Azolla*. Starch grains are also very rare although abundant in adjacent outer cortical and root cap cells.

By 300μ from the quiescent centre/root cap junction the triarch pattern is very distinct (Fig.4:1:19c). Phloem cells have a small cross-sectional area and a characteristic pattern of orientation as described in section 1 B ii a. The cells do not appear to have formed new vacuoles and have a low rate of fusion and inflation, fusion being either absent or extremely rare in protophloem, and therefore cells have only a few very small vacuoles. Protophloem sieve elements may be observed at the initial stages of cytological differentiation (Fig. 4:1:4d and 4:1:19c) as thick-walled pentagonal or diamond-shaped cells with low electron-density of cytoplasm. Single elements are initiated successively in a central position of the outermost layer in each phloem arc.

Metaphloem cells occupied the inner area of the phloem arcs, appearing to have similar cytoplasmic contents at this stage to protophloem cells although varying in cell size and shape. The first metaphloem sieve elements appeared to differentiate shortly after 600μ from the quiescent centre, initially in the inner boundary and later in the outer area of the phloem arcs. The forming metaphloem sieve elements initially developed a highly electron-dense cytoplasmic matrix with very large elongated highly electron-dense plastids. Vacuolar ontogeny was observed at this stage and was rapidly followed by inflation and a brief period of apparently selective autophagy.

Protoxylem cells may be distinguished between the phloem arcs at an early stage as larger, more radially elongated cells than phloem, with a higher rate of vacuolar fusion and inflation. Enlargement and vacuolation of protoxylem occurs slightly later than in metaxylem cells.

Cells may therefore be clearly distinguished by structural and ultrastructural characteristics immediately adjacent to the quiescent centre. Tissues developing later such as endodermis and pericycle may be identified long before cells in that tissue have ceased dividing.

The level and order of tissue differentiation at the cytological level were estimated as described in table 4:1:1. The results correspond, with some variations, to observations on the level and order of differentiation in other species. Measurements are given from the top of the root cap columella.

1. Root cap, cortex 0μ; 2. Procambium 40μ; 3. Metaxylem 120μ; 4. Vascular cambium, endodermis, pericycle 125μ; 5. Phloem, protoxylem 200μ. These results differ from those of Popham (1955) for pea and of Peterson (1967) for white mustard root who assessed the order of differentiation to be 1. Endodermis, 2. Pericycle, 3. Xylem, 4. Phloem. These differences are due to including the cortex, procambium and vascular cambium as tissues, and to altering the criterion of phloem differentiation from maturation or differentiation of the first sieve tube to differentiation of the phloem arcs, and to altering the criterion of cessation of cell division for the differentiation of endodermis and pericycle to visible differentiation of cells in those layers.

Maturation, or 'continued differentiation', of cells continued throughout the entire 20mm from the root apex. Protophloem sieve elements were the first cells to mature, being thick-walled anucleate cells containing very little stainable material with the exception of mitochondria, plastids and membranes. Metaphloem sieve elements mature shortly afterwards, being very similar to protophloem sieve elements with the exception that they are larger and have thinner walls.

By 20mm from the apex, some protoxylem cells have matured, having undergone secondary wall thickening and lignification, and autolysis of cytoplasmic contents. Phloem fibres have developed secondary wall thickenings and have a dense peripheral cytoplasm, but do not usually become lignified until 22-23mm from the tip.

Deposition of suberin in the Casparian strip was not observed in endodermal cells by 25mm from the apex.

Lateral root formation has not occurred by 30mm from the apex although cells adjacent to the protoxylem poles are still meristematic, contributing cells to the slowly growing primordia.

E. Summary, and criticism of the classical theory of root development

There is some controversy as to whether successive zones of division, expansion and maturation exist in the root apex and whether this is a valid and useful concept. This is very important from the developmental point of view as Brown (1963),

for example, has proposed a model of development and differentiation based on this concept.

The results presented here contrast sharply with the classical concept of zonation as viewed, for example, by Brown and Broadbent (1950) and Chaly and Setterfield (1975) for pea roots. In this view the apical 1-2mm of pea root consists entirely of non-vacuolated, meristematic cells (with the exception of root cap cells), while vacuolation and expansion take place in the following sections. Cell expansion then stops and cells mature without further division or expansion. Brown (1963) extends this view of zonation to include the feature that differentiation cannot commence until cell divisions are completed.

The discrepancies in the results and opinions arise largely as a result of the methods of analysis, in which values are either obtained by zonal analysis providing average values obtained from widely differing cells, or by more detailed analysis from anatomical studies. The limitations of these approaches have been discussed in chapter 1, and will be further discussed in chapter 4 section 2 and chapter 5.

The results therefore support the view that the classical concept of zonation is not valid (Popham, 1955; Peterson, 1967; Jensen, 1958; Goodwin and Stepka, 1945), and that it may be very misleading both in terms of understanding development, and in construction of experiments to investigate developmental features. The results were also inconsistent with several of the features of the model of root differentiation proposed by Brown (1963). The observations that were inconsistent with this model and with the classical concept of zonation are listed below. A summary of other features particularly relevant to development in the root apex is also included.

1. Differentiation of cells is apparent within the quiescent centre, and may be recognised at both the structural and ultrastructural levels. Differentiation therefore occurs at an extremely early stage of cell development and within the apical meristem. This is inconsistent with the view that differentiation does not commence until division ceases, and that division and differentiation involve qualitatively different, and incompatible, metabolic states.

2. Differentiation at the structural level may be identified by characteristics of cell shape and size, by the rate and degree of vacuolation and the size and number of vacuoles.

3. Cell shape and size largely reflect the pattern of frequency and orientation of cell division.

4. Characteristics of frequency and orientation of cell division vary within and between tissues. Characteristics of division may therefore reflect or be an integral part of differentiation.

5. Elongation is observed immediately adjacent to the quiescent centre/root cap junction, and increase in cell length in the apical meristem more than compensates for cell division. This is inconsistent with the view that a transition from a dividing to an elongating state occurs beyond the meristem.

6. Considerable radial expansion, including cell expansion in cross-sectional area, occurs in the apical meristem.

7. Cells may increase up to 6.5-fold in volume within 500μ of the quiescent centre. This is inconsistent with the view that cells cannot 'grow' in the meristem.

8. Radial expansion continues throughout the entire apical 20mm and is not confined to a zone between the 'dividing' and 'maturing' zones.

9. Areas of elongation and expansion in cross-sectional area do not correspond although they do overlap. Expansion proceeds more rapidly nearer to the apex than elongation, and continues at a further distance from the apex than elongation.

10. Expansion in root cross-sectional area does not have a smooth radial gradient, and tensions therefore arise in radial and tangential directions. This appears to be reflected in change in proportion of tissues making up the root. This compensation for tension induced within the root may be achieved by alteration of growth rate, polarity of growth, or by increase in volume of intercellular spaces.

11. Cell shape and size vary considerably between tissues and within a tissue at the same transverse level. Variation continues throughout the apical 20mm, in some cases in the absence of cell division. Change in cell shape which occurs in the absence of cell division may be either a reflection or a result of localised tensions resulting from differential growth, and may involve differential expansion, polarity of growth, or increase in intercellular spaces depending on the tissue concerned.

12. A negative coordination rather than synchrony is achieved between cells to maintain root shape in the transverse plane.

13. Elongation rate/unit length of root is coordinated throughout all cell layers, and synchrony of elongation is achieved between cells to maintain root shape in the longitudinal plane. Unlike cell expansion, elongation may therefore not involve tensions between cells in different tissues. Alternately, elongation may indeed be strictly restrained, and any inherent differences in growth rate may be reflected in differential radial expansion if radial expansion were not so strictly limited.

14. Elongation and expansion may therefore be under different or partially different control mechanisms, or different limiting conditions.

15. Cell division continues throughout the entire 20mm. Cell division is most frequent in the apical meristem, and continues for longest in outer stelar cells, particularly phloem. Cell division thereafter is confined to localised division in endodermis, pericycle, and perhaps inner cortical cells adjacent to protoxylem arcs in the formation of lateral root primordia.

16. Divisions involved in formation of lateral root primordia appear to be initiated within 500μ of the quiescent centre/root cap junction. Divisions are most frequent 2-4mm from the root apex, some distance beyond the apical meristem.

17. Cell division is therefore not confined to the apical meristem, although it appears (in normal circumstances) to be

confined to initial stages of cell development.

18. Division, cell expansion and differentiation therefore occur at all levels and appear to be intimately linked.

19. Differentiation between tissues at the structural level with respect to vacuole development may be attributed to different characteristics of vacuole ontogeny, fusion, expansion and development in different cell types at the ultrastructural level.

20. Differences in vacuolar ontogeny, and the rate and extent of vacuolar fusion and expansion may be observed immediately adjacent to the quiescent centre/root cap junction. These observations and others, particularly on plastid development, and starch and lipid content, indicate that differentiation is observed at the ultrastructural level within and immediately adjacent to the quiescent centre at the same level as structural differentiation may be observed. This is inconsistent with the view that differentiation does not proceed in dividing cells of the apical meristem.

21. Ultrastructural studies showed that differences in plastids, mitochondria, concentration of ribosomes in the cytoplasm, starch and lipid content, and characteristics of controlled cytoplasmic (and nuclear) breakdown are highly characteristic of cells as they emerge at the structural level. Controlled breakdown of cellular material appears to be a major feature of differentiation.

22. Major features of vacuole development include autophagy in cortical, endodermal, epidermal and pericycle cells; selective autophagy in metaphloem sieve elements; tonoplast breakdown in xylem and root cap, and probably in protophloem sieve elements; reduction in size to the size of E.R. cisternae or breakdown of the tonoplast in metaphloem sieve elements. The vacuole may therefore have a prominent role in both the mechanism and selectivity of breakdown of cellular material during differentiation. Vacuoles may therefore be more active in differentiation than is generally recognised.

23. Vacuole expansion may occur either by fusion, inflation and expansion, or by one or any combination of these, in varying degrees according to cell type. Expansion may be regulated in some cases by autophagy, or by storage or breakdown of osmotically active material.

24. Vacuolar ontogeny does not occur in root cap cells or in various cells arising from the procambium. In these cases, vacuoles appear to be inherited from the quiescent centre.

25. Vacuole expansion is not concurrent with cell expansion, as vacuoles may deflate, as in the case of root cap and metaphloem sieve element cells, during cell expansion; as vacuoles may not expand to the same extent as cell expansion; and as vacuolar expansion may not occur at the same distance from the apex as cell expansion as in protophloem, metaphloem and pericycle cells. Vacuolar expansion is therefore not linked necessarily and causally to cell expansion. Cell expansion consists of differential growth of vacuolar and cytoplasmic compartments, depending on cell type.

26. The level and order of differentiation were judged by different criteria from the more generally accepted criteria. Slight differences in the order of tissue differentiation were therefore obtained. Emphasis was placed on the observation that cells commence differentiation nearer to the apex than the level at which they are judged to have 'differentiated'.

It is clear from the above observations that cell division, expansion and differentiation occur at all levels in the root apex, and appear to a large extent to be interdependent. The classical concept of zones of division, expansion and differentiation in the root apex is not valid and is highly misleading both in terms of understanding development and of construction of experiments to investigate cell differentiation. The implications of these observations will be discussed in chapter 5.

2. BIOCHEMICAL ANALYSIS OF CELL DEVELOPMENT IN THE ROOT APEX

A. Introduction

In this preliminary study of gene expression in the root tip, protein and RNA composition during differentiation were examined.

In studies of the biochemistry of cell development in the root tip, the serial sectioning technique of Brown and Broadbent (1950) has become widely accepted owing to the fact that cells become progressively more advanced in their development with increasing distance from the apex. Although acknowledging that the sections do not contain a uniform population of cells, Brown (1963) claims that 'if the course of development in the different cell layers were highly variable ... no consistent trend would be shown by the data ... (however) as the series of values always shows a well-defined pattern of change, it may therefore be accepted that it is probable that cells in all layers traverse a similar development ... that is common to all tissues in the growing zone.'

This is highly questionable. Indeed, the scatter of points around the curves drawn in many of the graphs for protein content, enzyme activities and respiration rate obtained by Brown and his co-workers (for example Robinson and Brown, 1952; Brown and Broadbent, 1950; Brown, 1963) indicate that there are not such consistent trends, and that lack of uniformity might well be attributable to a different course of development between tissues.

Studies with the light and electron microscopes (chapter 4 section 1) indicated that zonal analysis would be only of very limited value in the investigation of the development of different tissues. As can be seen in figure 4:1:10, tissues alter considerably in their proportions throughout the root tip. In particular the root cap, which contributes more than 50% of the volume of the apical millimetre and more than 10% of the subsequent three millimetres is not involved in the developmental sequence of the root proper, although contributing a substantial amount of tissue to the apical two millimetres. Furthermore,

it consists of cells in all stages of development from the initial to the fully mature state. The cortex, which varies from 0% in the apical 400μ to about 50% immediately beyond the quiescent centre, rises to more than 80% of tissue volume by 6mm. This tissue might therefore be expected to dominate the results obtained from biochemical analyses, although to a varying degree depending on distance from the apex. Biochemical development of other tissues, which each contribute much less than 10% of the total volume of the root including endodermis, pericycle, epidermis and different types of phloem and xylem in the stele, will be obscured.

Histochemical studies also indicate that variations between tissues in enzyme activities do occur both in the timing of the change in activity and in the extent of activity, and that activity of certain enzymes may not be detectable at all in some tissues (Jensen, 1955). Despite the objections that may be raised to these methods, it is clear that in analysis by serial sectioning differences in the development of enzyme activity in tissues other than the cortex would be represented as very minor variations in the overall trend, that absence of enzyme activity in one or more minor tissues would not be detected, and that enzyme activity in one minor tissue that was absent in others would probably be too low to be detectable.

In addition, therefore, to analysing protein and RNA in entire segments, estimates were also obtained where possible for smaller zones dissected from these segments. Dissection was carried out with the aid of a dissecting microscope, sections being separated into two main zones. Segments were normally dissected into A. 'Cortex' which consisted of cortex and epidermis, and in the apical 4mm also the root cap; and B. 'Stele' which included xylem, phloem, endodermis and pericycle. For two-dimensional analysis of protein, sections were either dissected as above, or were divided into C. 'Cortex/stele' which consisted of all tissues except the epidermis and root cap, and D. 'Epidermis' which consisted of epidermis, probably some outer cortical cells, and the root cap in the apical 4mm. Unfortunately, it was not feasible to obtain these sections for work with calmodulin, NAD kinase and RNA owing to the number of sections

required, and in the case of NAD kinase and RNA also to the length of time required for the dissection. Admittedly the separation of tissues achieved was very limited, however, I felt it was necessary to obtain some indication as to whether differences in protein composition and development of the pattern of protein composition in early differentiation might occur, or whether all tissues traversed the same basic course of development at the same distance from the apex as viewed by Brown (1963).

B. Developmental changes in protein and RNA content

(i) Protein content

Protein concentration was estimated by the Lowry method and the Bradford method as described in chapter 2 section 2E. The results are shown in figures 4:2:1. Total protein/section and protein concentration/section (Fig.4:2:1a) were both observed to be very low in the root cap, increasing through the zone containing the quiescent centre with protein concentration rising rapidly to a maximum between 700 and 1000μ from the root tip, and total protein to a maximum 1-2mm from the root tip. This was followed by an equally rapid fall to a minimum between 3-5mm from the tip as cells enlarged and the vacuome occupied an increasing proportion of most cells. After 5-6mm protein content remained approximately constant.

Protein/cell did not, however, vary so greatly (Figure 4:2:1b). The protein content of cells in the quiescent centre zone was again low, but rose briefly in the dividing cells on either side, probably due to an increase of protein for production of daughter cells increasing the amount of protein/cell prior to division. Protein content then fell slightly to a minimum 2-3mm from the apex. A two-fold increase in protein content/cell then occurred between 3 and 6mm as cells expanded rapidly in volume, and then fell slightly as cell expansion decreased considerably but the vacuome fraction and programmed cell degeneration continued to increase. The results obtained by the Lowry and Bradford methods were very similar.

Figure 4:2:1. Developmental changes in protein content during differentiation in the root apex

Peas were grown as described in chapter 2 section 2 B ii a and were harvested after 65 hours. One millimetre sections were then dissected, and protein assayed either by the Lowry method (figures a and b), or by the Bradford method (figures c and d).

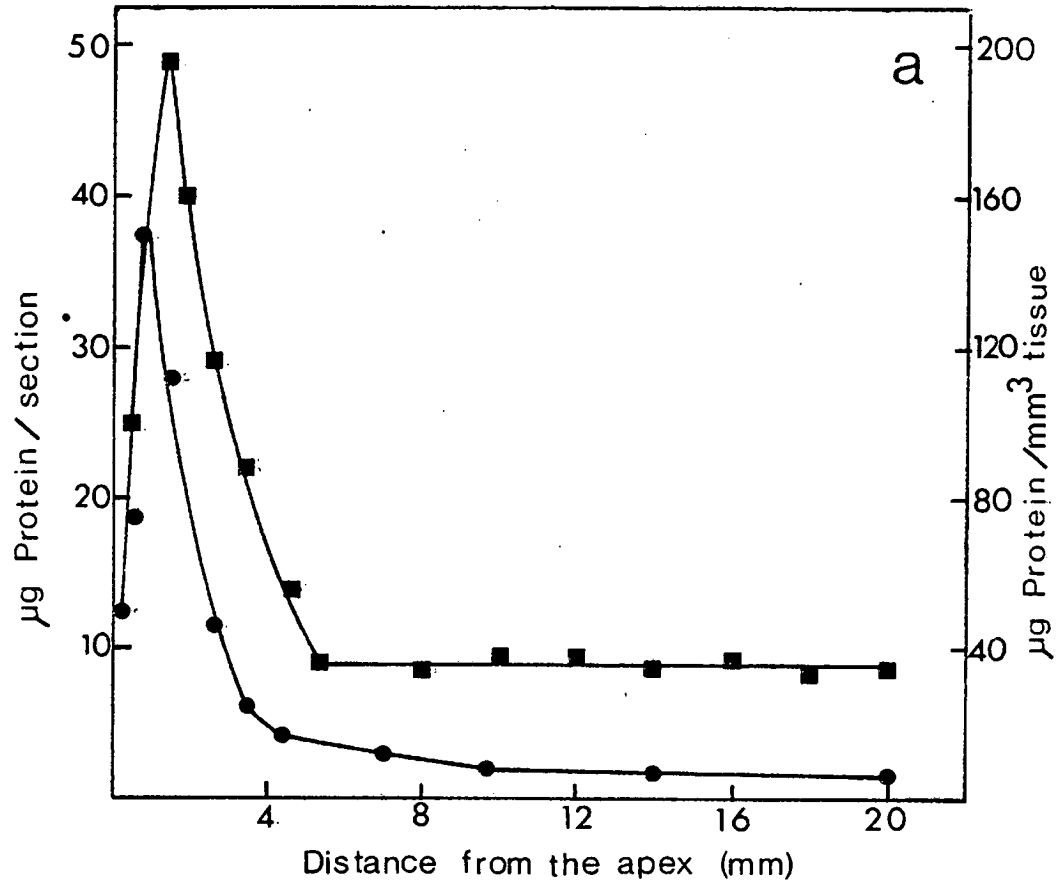
a) Developmental changes in protein content (—■—) and protein concentration (—●—) per section.

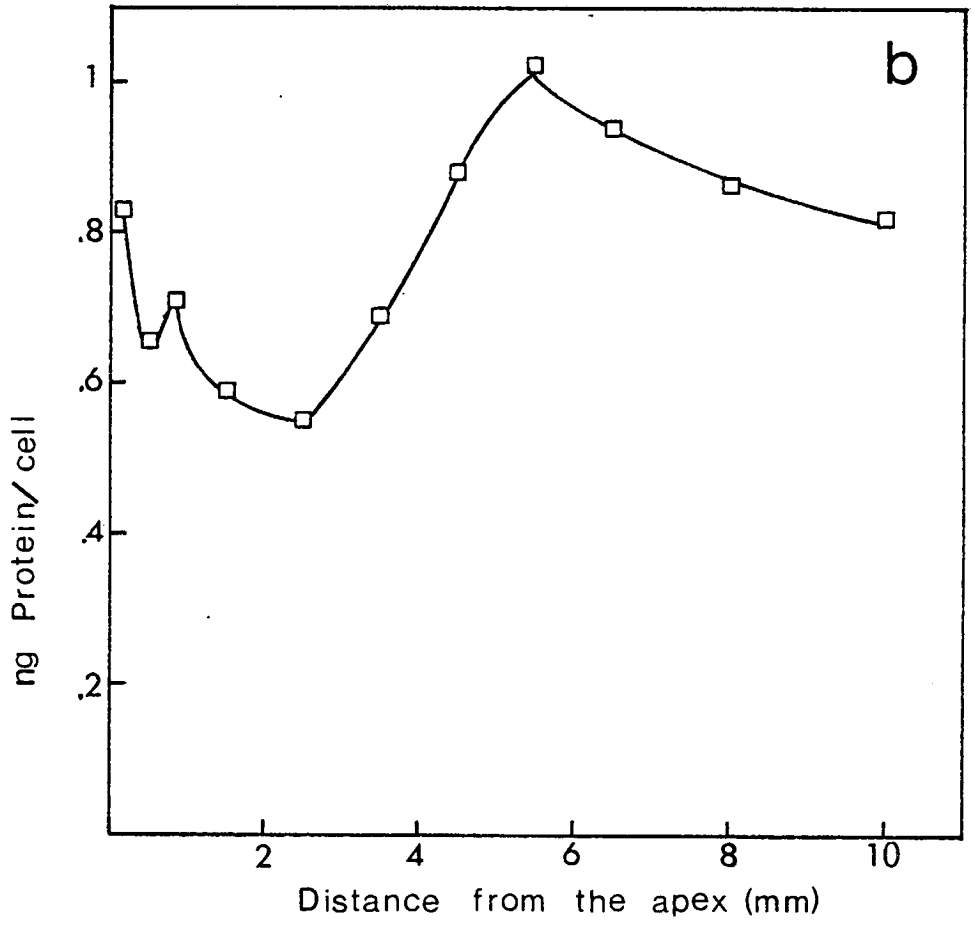
b) Developmental changes in protein content per cell (—□—).

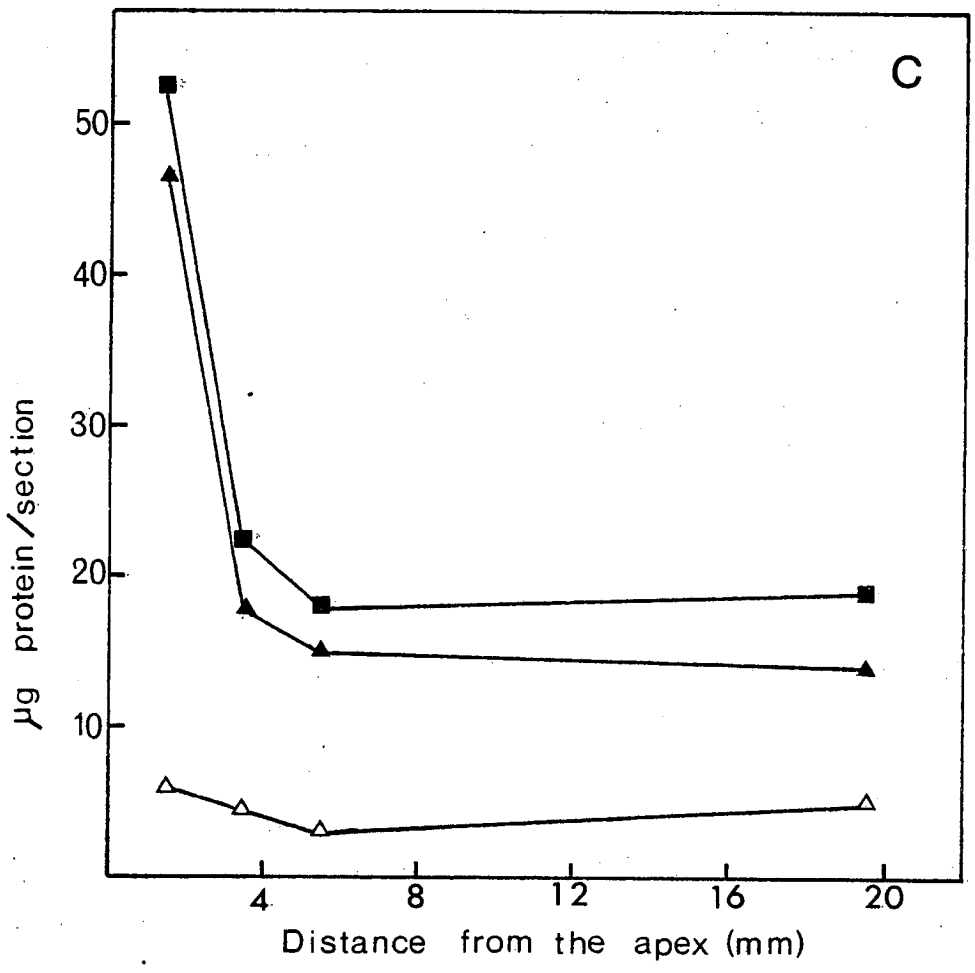
c) Developmental changes in total (—■—), cortical (—▲—), and stelar (—△—) protein content per section.

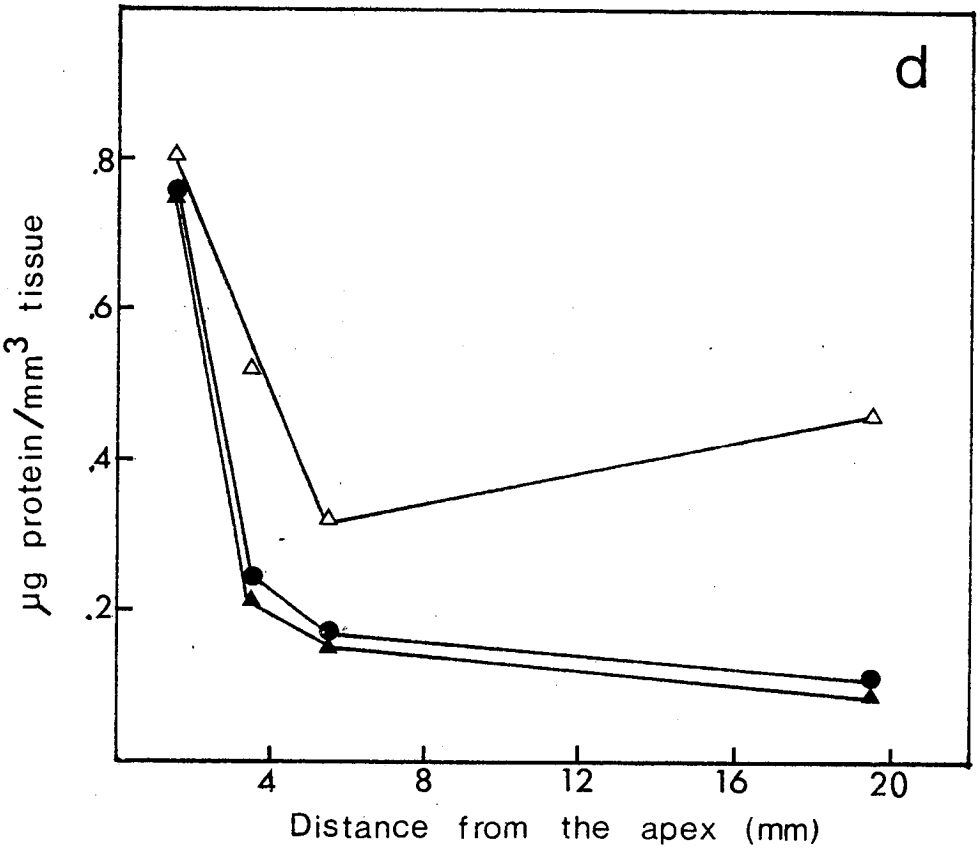
d) Developmental changes in total (—●—), cortical (—▲—) and stelar (—△—) protein concentration.

Fig. 4:2:1.


a
50
40
30
20
10
200
160
120
80
40
µg Protein / section
µg Protein /mm³ tissue
4
8
12
16
20
Distance from the apex (mm)


b
1
.8
.6
.4
.2
ng Protein/ cell
2
4
6
8
10
Distance from the apex (mm)


c
50
40
30
20
10
µg protein/section
4
8
12
16
20
Distance from the apex (mm)


d
.8
.6
.4
.2
µg protein/mm3 tissue
4
8
12
16
20
Distance from the apex (mm)

The results obtained for total protein/section followed a similar trend to those obtained for pea root by Brown and Broadbent (1950) and Heyes (1960). However, although a two-fold order of increase in protein/cell between 1 and 8mm from the tip is in reasonable agreement with the data of Heyes, a marked rise of a ten-fold increase in protein/cell from the apex to 6mm as described by Brown and Broadbent was not detected. This discrepancy is due to the differences in the estimates of protein/section, as cell number estimates were very similar. The estimate of protein content/cell varied from 28 times higher than that obtained by Brown and Broadbent for zones of low protein content to three times higher in zones of higher protein content. The estimates that I obtained for protein content were similar to those of Heyes. Although both Heyes and Brown and Broadbent used the Kjeldahl method for nitrogen, Heyes used 10% TCA or 80% boiling ethanol to precipitate protein whereas Brown and Broadbent used only 2.5% TCA. It therefore seems probable that the 2.5% TCA concentration used by Brown and Broadbent was insufficient to precipitate the protein completely, in particular at lower protein concentrations.

The very low increase in protein/cell, from an average of about 720pg in meristematic cells to 820pg in cells 10mm from the apex, in conjunction with a much greater (thirty-fold) increase in cell volume resulted in a considerable reduction in protein concentration/cell during early differentiation. Although I did not obtain quantitative data for the proportion of cytoplasm in cells, nor did I investigate the concentration of protein in the vacuole and cytoplasmic fractions, it is probable that this represented a substantial reduction in concentration of cytoplasmic protein. The results are therefore not in agreement with those of Brown and Broadbent (1950) who concluded that protein content in meristematic cells was considerably lower than in fully vacuolated cells, and that protein concentration/cell decreased only slightly during differentiation in the apical 10mm.

When protein was further analysed in cortical and stelar tissues by the Bradford method, it was found that although stelar protein contributed very little protein to tissues in

the apical 20mm, it occupied an increasingly larger proportion of total protein content owing to a relatively higher protein concentration (Fig.4:2:1c and d). In the initial stages of development 1-2mm from the apex, stelar protein constituted almost 12% of total protein of the root. As this section also occupied almost 12% of the root volume, the protein concentrations of the stelar and cortical fractions were very similar. However, as the protein content of the stele altered very little after that, and tissue volume did not increase substantially, the protein concentration did not fall to any great extent. A sharper drop in the concentration of cortical protein occurred owing to both a considerable drop in protein/section and a relatively greater increase in cross-sectional area than the stele resulting in a greater proportion of the root being occupied by cortical tissue.

By 10-20mm from the root tip, the concentration of protein in the stele was 5-6 times as high as that in the cortex, and thus although occupying only 7% of the volume of the root, it constituted more than 25% of the total protein. Although many cells in the stele have undergone considerable selective or complete degeneration by this stage, the higher protein concentration may perhaps reflect a greater amount of structural protein in secondary cell walls and a lower vacuome content of many non-xylem cells than cortical cells.

(ii) *In vivo* incorporation of (^{35}S) methionine into protein

The pattern of uptake of (^{35}S) methionine into protein (Figure 4:2:2) during differentiation after *in vivo* labelling as described in chapter 2 section 2F was found to be completely different from that of protein content. Incorporation into protein/cell or /µg protein was highest in the apical mm, falling rapidly by 2mm, with a second smaller peak 2-4mm from the tip before a rapid fall to virtually no incorporation into protein by 10mm. On analysis of incorporation in the cortex and stele, it was found that the specific activity of incorporation into protein fell rapidly in the stele, while the increase observed between 2 and 4mm over the whole root was due to an increase in incorporation in the cortical section alone (Figure 4:2:2b).

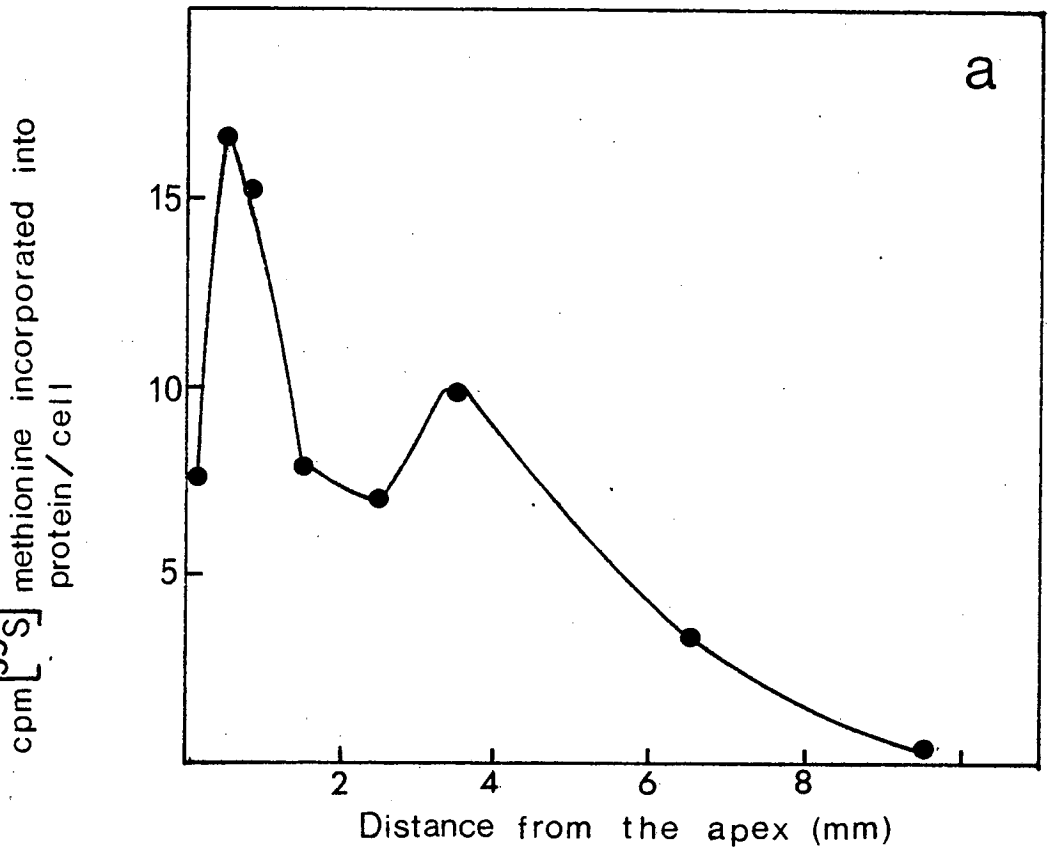
Figure 4:2:2. Developmental changes in the incorporation of (^{35}S) methionine into protein in the root apex

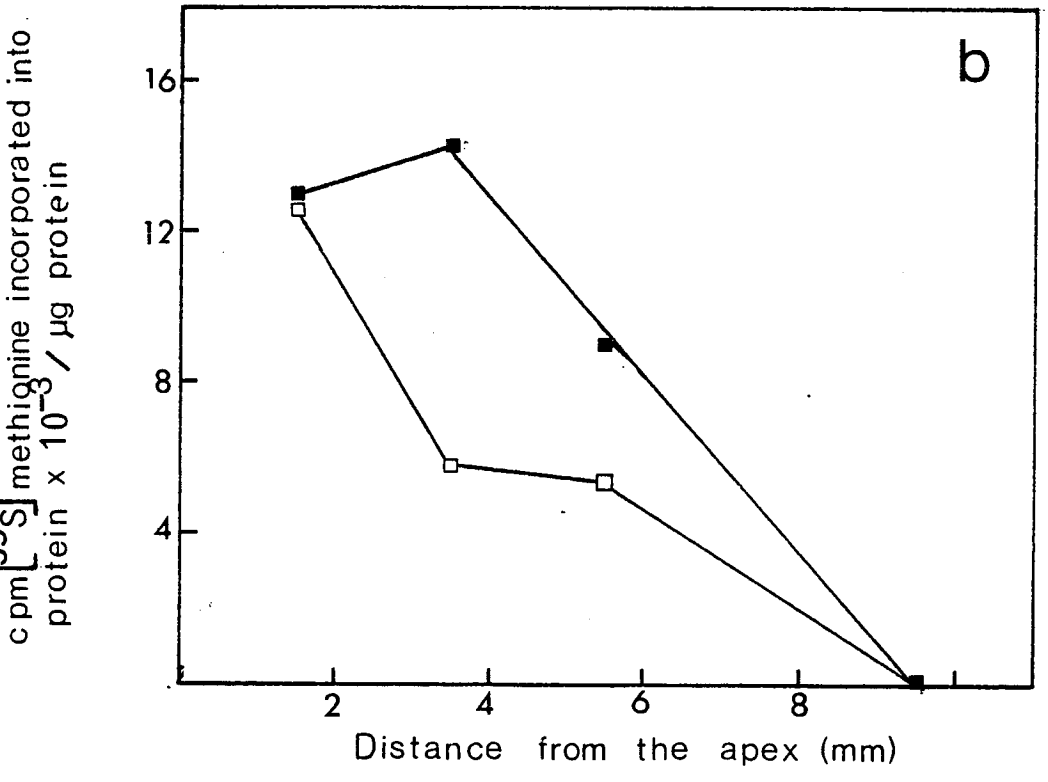
Roots were grown as described in chapter 2 section 2 B ii a and were harvested after 65 hours. Roots were then labelled for two hours with (^{35}S) methionine as described in chapter 2 section 2 F, dissected into one millimetre serial sections, and the amount of (^{35}S) methionine incorporated into protein assayed essentially according to the procedure of Mans and Novelli (1961).

a) Developmental changes in incorporation of (^{35}S) methionine into protein per cell.

b) Developmental changes in incorporation of (^{35}S) methionine into protein per unit protein in cortical (——■——) and stelar (——□——) tissues.

Fig. 4:2:2.


a
15
10
5
2
4
6
8
Distance from the apex (mm)
cpm [35S] methionine incorporated into protein / cell


b
16
12
8
4
2
4
6
8
Distance from the apex (mm)
cpm [35S] methionine incorporated into protein x 10⁻³ / µg protein

Incorporation of (^{35}S) methionine into the root was not limited by the amount of (^{35}S) methionine supplied in any of the sections.

Incorporation of exogenously supplied amino acids into protein to some extent may be used as a measure of *in vivo* protein synthesis. However, the incorporation into protein will depend on the size of the metabolic amino acid pool for that amino acid, the availability of the precursor for protein synthesis relative to the availability of the endogenous precursor, the rate of protein degradation and the amino acid composition of protein (Davies, 1980). Experiments carried out on maize root tips (Oaks, 1965a and b) indicate that uptake of exogenously supplied amino acids is very rapid, and that incorporation into protein becomes linear after a time lag of only six minutes which was suggested to be the time required to equilibrate with the amino acid pool. The endogenous availability of exogenously supplied amino acids is therefore probably not limiting in *in vivo* labelling experiments which were carried out for two hours. Oaks also obtained evidence that the sizes of the amino acid pools in different zones of the root tip were regulated by the level of protein synthesis, and that synthesis and transport of an individual amino acid was regulated by the amount of that particular amino acid in the pool. The amino acid pool size therefore appeared to be proportional to requirements throughout the root tip and to be regulated by requirements. Local uptake of (^{35}S) methionine in pea root tips might therefore be expected to reflect protein synthesis, and the ratio of (^{35}S) methionine to endogenous methionine in the amino acid pool to initially remain constant. The initial uptake of (^{35}S) methionine into proteins should therefore be proportional to protein synthesis.

These observations indicate that incorporation of exogenously supplied amino acids into protein in root tips as described for pea roots may be taken as a rough estimate of the rate of protein synthesis. This was supported indirectly by the observation that ribosomes, probably largely in the form of polysomes followed a similar pattern to *in vivo* labelling of protein being very high in the apex (for example figure 4:1::12)

decreasingly progressively further from the apex (for example figure 4:1:14a and d, 4:1:16a), as has also been observed by Chaly and Setterfield (1975).

If the values obtained may therefore be taken as an indication of protein synthesis, it is interesting to note that protein content and protein synthesis do not correspond; protein content/cell increasing while protein synthesis/cell is decreasing (compare figure 4:2:1b and 4:2:2a).

The high level of protein synthesis in the apex, and to a lesser extent in the cortex 2-4mm from the tip, probably reflects in part the synthesis of proteins for newly forming cells thus increasing the average value/cell as cells in all stages of division were included in the measurements. However, it seems probable that many proteins synthesised in the apex during division and early stages of differentiation may also have a high turnover rate. As the cells mature and the vacuome occupies an increasingly large proportion of cortical cells (which provide about 80% of the root volume) while an increasing number of stelar cells develop secondary walls, a progressively higher proportion of protein may have a low turnover rate such as structural protein. Protein would therefore accumulate in the cell without concurrent protein synthesis.

It is interesting that the high apparent rate of protein synthesis in the cortex 2-4mm from the tip coincided with the zone of very active vacuolar autophagy (chapter 4, section 1 C i b I) as well as the zone of active cell division in the developing lateral root primordia in the inner cortex (chapter 4, section 1 B i c). Autophagy might well act as a mechanism for rapid turnover of protein, allowing rapid alteration in composition of cytoplasm of both protein and non-protein materials, both as a result of immediate isolation of material of the ground plasm and of degeneration of the contents potentially providing materials for synthesis of new components.

The reduction in specific activity of protein synthesis in stelar tissue 2-4mm from the tip coincided with the area of formation of metaphloem sieve elements (chapter 4, section 1 C b IV). Although the formation of these cells appeared to involve a phase of selective autophagy, this was brief, and appeared to

be linked with cytoplasmic and nuclear degeneration. As differentiation therefore involved breakdown of the nucleus as well as much of the cytoplasmic protein synthesising machinery, synthesis of nuclear-encoded proteins would fall even if long-lived mRNA species were synthesised before nuclear breakdown. The earlier fall in protein synthesis in the stele than the cortex despite its higher protein concentration therefore probably reflects the fall in synthesis of nuclear-encoded proteins, with an increasing proportion of total protein occurring as structural protein in thickened walls of the developing sieve elements, and proteins with a low turnover rate in the sieve element matrix.

(iii) RNA content

Nucleic acids were extracted from serial 1.25mm segments along the root, and messenger + ribosomal RNA was separated from this fraction as described in chapter 2 section 2 J i and chapter 3 section 4 A. Rough estimates of the amount of m+rRNA in different sections were obtained, and the results are shown in figure 4:2:3a and b. RNA/section (which consisted almost entirely of rRNA) was found to follow a similar pattern to that of protein/section, increasing to a maximum 1-2mm from the root tip, then falling to a minimum by 6mm. RNA/cell, however, was at a minimum in the apical millimetre and increased approximately 2.5-fold throughout the apical 10mm. A similar increase in RNA/cell has been observed in roots of other species as well as in pea roots using a variety of extraction procedures and microfluorometric techniques (Heyes, 1960; Sunderland and McLeish, 1961; Jensen, 1958; Bucknall and Sutcliffe, 1965). This increase in RNA/cell might be due either to increased transcription of RNA, to a decrease in its degradation, or to an accumulation of RNA with age due to a long half-life if its rate of synthesis were higher than the rate of degradation. As Chaly and Setterfield (1975) have observed that rRNA synthesis in the nucleus decreases with age in cortical cells, the increase in RNA/cell may reflect accumulation of RNA rather than increasing synthesis.

Owing to a 20-fold increase in average cell volume throughout

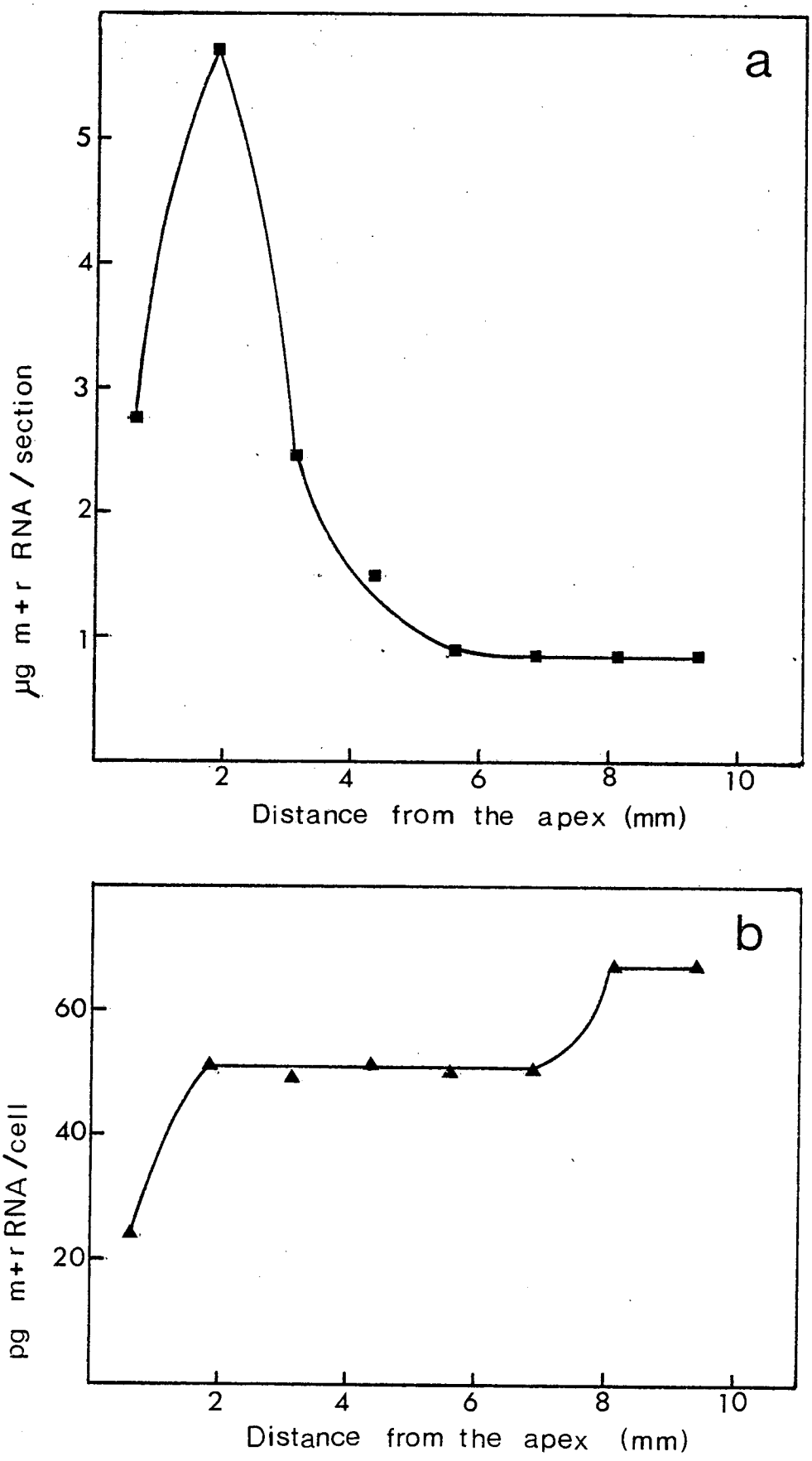
Figure 4:2:3. Developmental changes in RNA in the root apex

Nucleic acids were extracted from serial 1.25mm segments of pea root as described in chapter 2 section 2 J i. Messenger and ribosomal RNAs were precipitated from total nucleic acids by 2-3M potassium acetate.

a) Developmental changes in m + rRNA per section.

b) Developmental changes in m + rRNA per cell.

Fig. 4:2:3.


a
5
4
3
2
1
µg m+r RNA / section
2
4
6
8
10
Distance from the apex (mm)
b
60
40
20
pg m+r RNA /cell
2
4
6
8
10
Distance from the apex (mm)

the zones examined, the m+r RNA concentration/cell was lower in more basal sections. This is perhaps reflected in the decreasing concentration of ribosomes that occurred towards the base of the root. However, as I did not quantitate ribosome number, there may be a differential reduction in ribosome number and rRNA concentration.

Bucknall and Sutcliffe (1965) noted using microfluorimetry of thin sections that although the concentration of RNA/cell initially rose to about 2.5mm and continued to rise in outer stelar cells, that RNA concentration in cortical and metaxylem cells decreased beyond 2.5mm. It therefore appears that RNA synthesis or accumulation occurs to a greater extent, or for a longer time in outer stelar than in metaxylem or cortical cells.

(iv) Incorporation of (^{3}H) amino acids into protein during *in vitro* translation of mRNA

An increase in rRNA/cell is normally assumed to indicate an increase in capacity for protein synthesis. However, in the case of pea root, rRNA/cell increases while apparent protein synthesis/cell decreases. (Compare figure 4:2:3b with 4:2:2a). It therefore seemed possible that protein synthesis was limited by some other component of the protein synthesising mechanism, such as the amount of mRNA. The m + r RNA fraction obtained from successive sections of the root was therefore used to direct protein synthesis in a wheatgerm-S30 cell-free protein synthesising system as described in chapter 2 section 2 J ii-iv and chapter 3 section 4 B, and the rate of incorporation of (^{3}H) amino acids into protein used to estimate relative concentrations of mRNA during development.

The rate of incorporation of (^{3}H) amino acids into protein during *in vitro* translation was found to remain approximately constant in different zones of pea root per µg RNA. The amount of *in vitro* translatable mRNA/cell was therefore found to follow a similar trend to total m + r RNA/cell (Figure 4:2:4), increasing 3-4-fold throughout the apical 10mm.

As briefly described in chapter 3 section 4 B, the degree of *in vitro* translation may not reflect the amount of total or

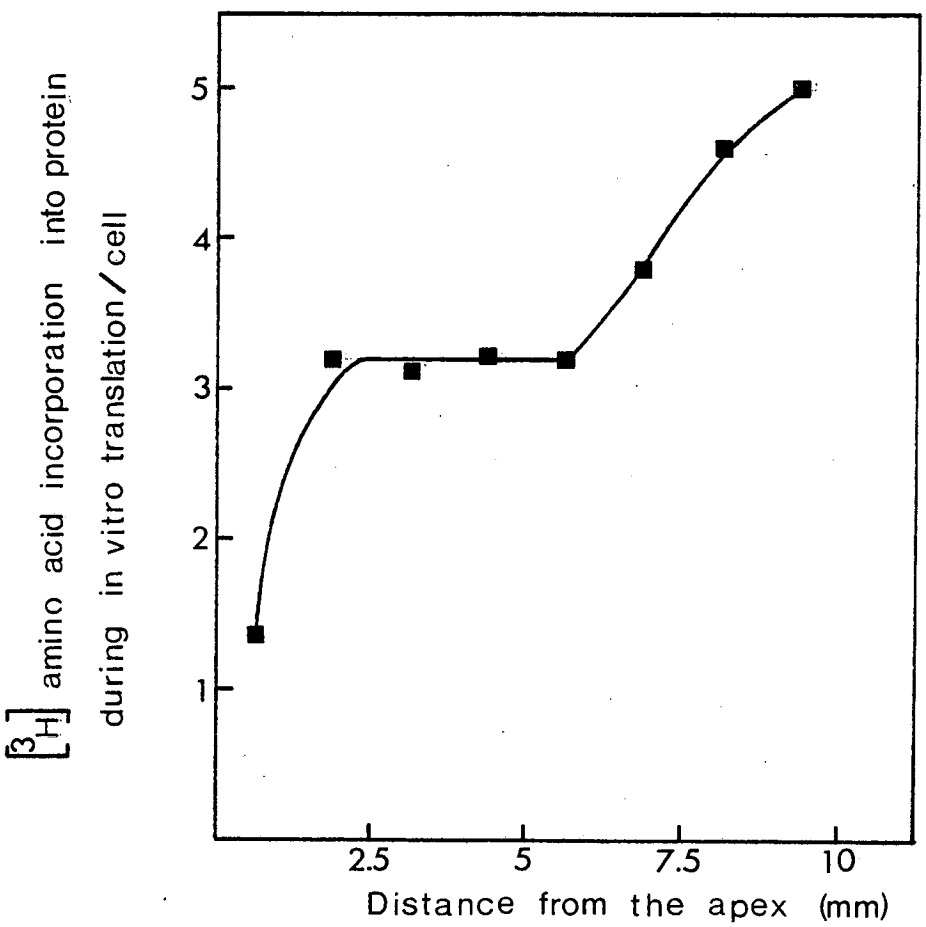
Figure 4:2:4. Developmental changes in *in vitro* translatable mRNA

Messenger + Ribosomal RNA was extracted from serial 1.25mm root sections as described in chapter 2 section 2 J. Equal amounts of RNA from each section were then used to direct protein synthesis in a wheatgerm S30 cell-free protein synthesising system as described in chapter 2 section 2 J ii-iv.

Incorporation of (^{3}H) amino acids into protein was then assayed according to Mans and Novelli (1961).

The results were adjusted for the concentration of m + rRNA per cell and are expressed as (^{3}H) amino acids incorporated into protein per cell.

Fig. 4:2:4.


[3H] amino acid incorporation into protein during in vitro translation / cell
5
4
3
2
1
2.5
5
7.5
10
Distance from the apex (mm)

individual input mRNAs, nor need the rate of *in vitro* translation bear any resemblance to the *in vivo* rate of protein synthesis. The comparison of mRNA concentration in different developmental zones by *in vitro* protein synthesis may therefore not be valid. However, as efficiency of mRNA translation varied with the amount of RNA added to the translation system (chapter 3 section 4 B; Shafritz, 1977) equal amounts of RNA from different regions of the root of a non-limiting concentration (7µg/50µl translation) were added to the translation system, so that efficiency of translation of RNA from different zones of the root might be comparable. Although RNA from different zones of the root might have different requirements for optimal translation, a similar efficiency of translation of RNA was also indicated by the observation that total incorporation of amino acids into protein was very similar if the same RNA concentration from different zones was used.

Differential extractability of RNA or mRNA might also result in artifactual differences in *in vitro* translatable mRNA between tissues. However, as estimates of the amount and developmental trends of total RNA/cell were similar to those obtained by a variety of other methods, and the trend of *in vitro* translatable mRNA was similar to that for m + rRNA concentration, differential extraction of mRNA or total RNA is perhaps not likely to significantly affect the results.

The relative rates of incorporation of (^{3}H) amino acids into protein during *in vitro* translation of mRNA obtained from different developmental zones as shown in figure 4:2:4 may therefore represent approximately the relative amounts of mRNA during development. As indicated above (section ii), estimates of *in vivo* protein synthesis are not likely to be affected by differences in either size or composition of amino acid pools in different developmental zones, and thus estimates of (^{35}S) methionine incorporation into protein probably reflect rates of *in vivo* protein synthesis.

It is therefore very interesting to note that whereas both *in vitro* translatable mRNA and rRNA/cell both continue to increase from 1-10mm (Figure 4:2:3b and 4:2:4), that there is a concurrent decrease in *in vivo* protein synthesis as measured by (^{35}S)

methionine incorporation into protein/cell (Figure 4:2:2a). By 9mm from the tip, protein synthesis appears to be negligible although the total amount of both messenger and ribosomal RNAs appear to be in non-limiting concentration.

It was therefore of interest to compare the *in vitro* translation products with *in vivo* labelled translation products and protein composition during differentiation to investigate whether products of *in vitro* protein synthesis resembled those of *in vivo* protein synthesis. Although in general the products could not be directly compared due presumably at least in part to lack of co- or post-translational modifications of *in vitro* translation products and possibly to misreading of mRNA and to reading of incompletely processed mRNA, the degree and the nature (qualitative or quantitative) of change in polypeptide composition *in vivo* could be compared with the degree and nature of change in *in vitro* translation products.

C. Quantitative and qualitative changes in protein and mRNA species during development

(i) Developmental changes in mRNA species as analysed by *in vitro* translation of mRNA

In vitro translation products of mRNA isolated from serial 1.25mm sections from 0-10mm and 18.75-20mm from the root tip were observed on two-dimensional polyacrylamide gels as described in chapter 2 section 2 J v and G. The results were striking. Translation products were almost identical throughout the entire 20mm examined. A few changes occurred within the apical 5mm as described in figure 4:2:5 while very little change, if any, occurred between 5 and 20mm from the root tip. Despite the objections raised in chapter 3 section 4 to the estimation of individual mRNAs by *in vitro* translation, the minimal degree of apparent change in *in vitro* translatable mRNA species in view of the considerable structural and ultrastructural changes that take place is in marked contrast to the generally-held assumption that differentiation is largely controlled at the level of transcription.

Figure 4:2:5. Developmental changes in *in vitro* translation products of pea root mRNA as analysed by two-dimensional polyacrylamide gel electrophoresis

Messenger + ribosomal RNA was extracted from serial 1.25mm pea root sections, and used to direct protein synthesis in a wheatgerm S30 cell-free protein synthesising system as described in chapter 2 section 2 J.

The polypeptides were then separated by NEPHGE in the 1st dimension and by SDS-PAGE in the 2nd dimension as described in chapter 2 section 2 G iii. The gels of the radioactively labelled *in vitro* translation products were then fluorographed as described by Bonner and Laskey (1974).

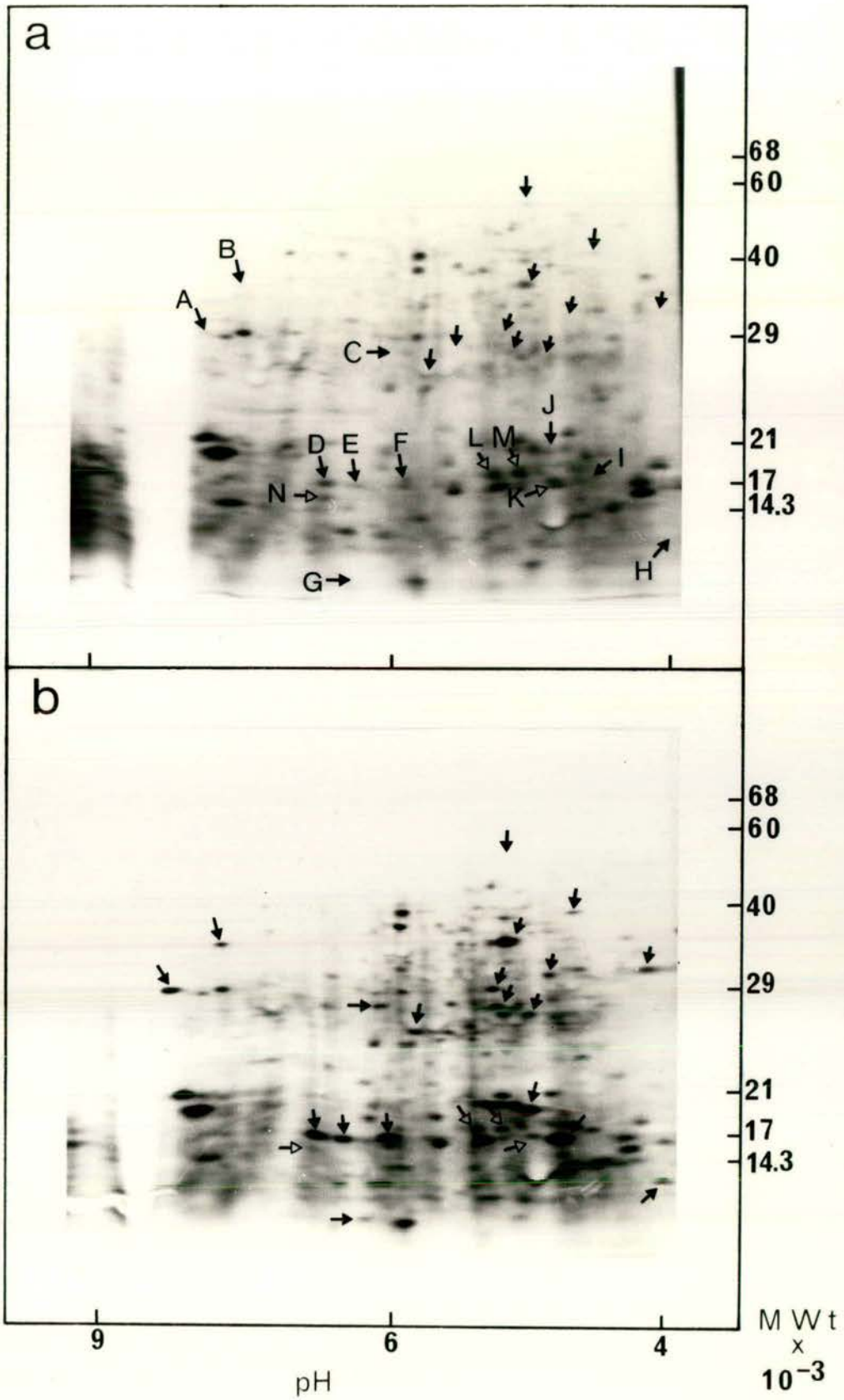
a) *In vitro* translation products of RNA obtained from sections 1.25-2.5mm from the apex.
b) *In vitro* translation products of RNA obtained from sections 8.75-10mm from the apex.
c) *In vitro* translation products of RNA electrophoresed in the presence of 10mM $CaCl_2$.
d) *In vitro* translation products of RNA electrophoresed in the presence of 15mM EGTA.

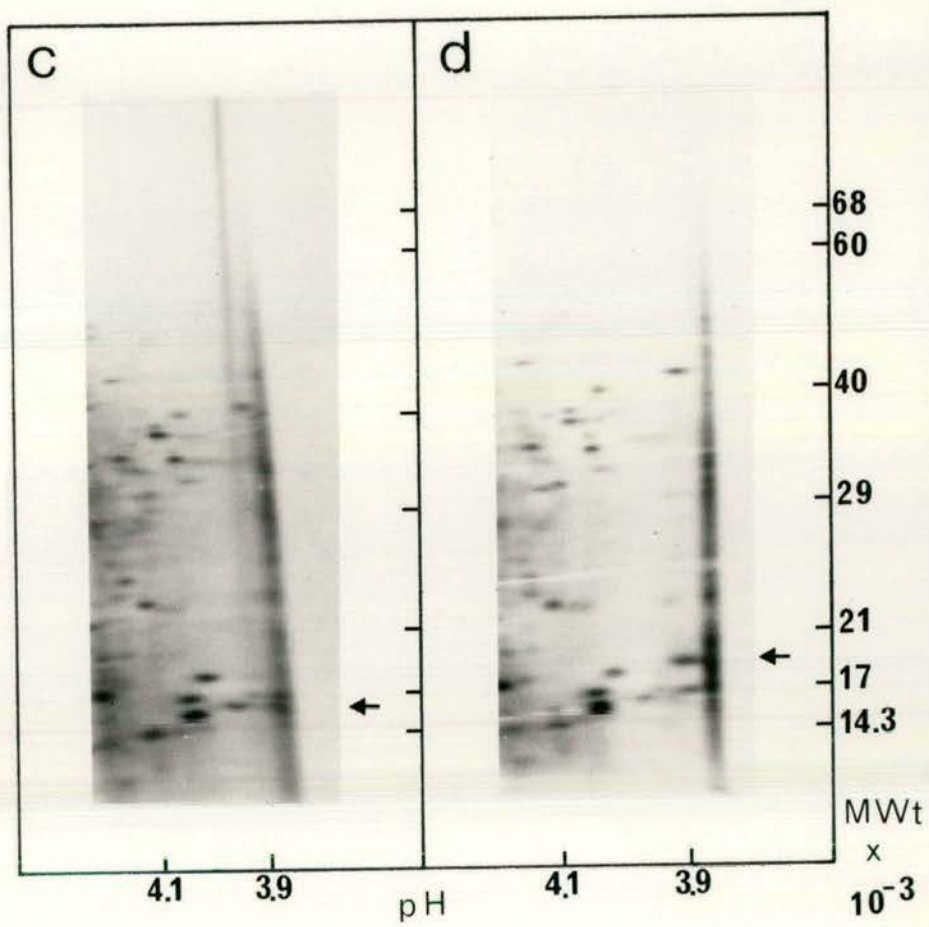
Approximately 25 *in vitro* translation products were observed to alter quantitatively or qualitatively within the apical 20 millimetres of the pea root apex. Developmental changes are listed below.

↙ *In vitro* translation products increase with distance from the apex
↗ *In vitro* translation products decrease with distance from the apex

A Not detectable 0-4mm from the apex. Prominent after 5mm
B Not detectable 0-2.5mm from the apex. Prominent after 4mm
C Not detectable 0-3.5mm from the apex. Prominent after 5mm
D Very low 0-1.25mm from the apex. Increases until 4mm after which it is very prominent.
E Very low 0-1.25mm from the apex. Increases until 4mm after which it is very prominent
F Very low 0-1.25mm from the apex. Increases until 4mm after which it is very prominent.
G Not detectable 0-3.5mm from the apex. Prominent after 5mm
H Not detectable 0-2.5mm from the apex. Prominent after 5mm
I Not detectable 0-3mm from the apex. Prominent after 4mm
J Not detectable 0-5mm from the apex. Prominent after 5mm
K Present 0-3mm from the apex. Not detectable after 4mm
L Prominent 0-4mm from the apex. Faint after 5mm
M Prominent 0-4mm from the apex. Faint after 5mm
N Prominent 0-4mm from the apex. Faint after 5mm

Fig. 4:2:5.




c
d
68
60
40
29
21
17
14.3
MWt
x
10⁻³
4.1
3.9
pH
4.1
3.9

It was possible that the similarity of translation products was due to an artifact of the *in vitro* translation system, which may, for example, have preferentially allowed translation of specific mRNAs. However, there was not much evidence for preferential translation under different translation conditions other than the commonly-observed increase in high molecular weight products with adjustment of ion concentrations as described in chapter 3 section 4, apparently due to prevention of premature termination. It was also possible that the mRNAs observed by this procedure (i.e. nuclear mRNAs, and relatively few mRNAs coding for high molecular weight polypeptides) were not those involved in differentiation; or that there were relatively few differences occurring as cells aged, although greater differences occurred between tissues.

It was also, however, possible that relatively few changes in protein composition occurred during differentiation. Protein composition was therefore analysed by two-dimensional polyacrylamide gel electrophoresis, and three proteins were further analysed by biological assay.

(ii) Developmental changes in protein composition and in the pattern of *in vivo* labelled proteins as analysed by two-dimensional polyacrylamide gel electrophoresis

In order to investigate further the apparent absence of any marked degree of alteration in mRNA composition during development as analysed by *in vitro* translation, the degree of qualitative and quantitative changes in protein composition and synthesis were examined by silver staining of proteins after two-dimensional polyacrylamide gel electrophoresis and by autoradiography of two-dimensional gels after *in vivo* labelling of roots with (^{35}S) methionine as described in chapter 2 section 2 F and G.

Comparison of protein composition in different developmental zones by two-dimensional polyacrylamide gel electrophoresis is dependent on an absence, or at least similarity of, modification of proteins; and on a similar degree of solubilisation of proteins in different zones. As described in chapter 3 section 1, care was taken to minimise artifacts during extraction and

electrophoresis of proteins, while strong denaturing conditions were employed in order not only to produce almost completely denatured proteins and thus reduce differential denaturation, but also to increase solubilisation of proteins, to reduce precipitation of extracted protein and prevent aggregation of ribonucleoprotein particles. Modification of protein was examined by comparison of the protein patterns obtained from sections homogenised separately and together. As no obvious differences from the expected pattern could be detected, it appeared that differential modification in different zones was probably not significant.

One millimetre serial sections were therefore obtained from pea root, and proteins separated by two-dimensional electrophoresis were examined. Considerable differences in protein composition and pattern of *in vivo* labelled polypeptides occurred between the apical region and the more basal regions. Proteins were therefore found to alter to a far greater extent both qualitatively and quantitatively than *in vitro* translatable mRNA during development. A summary of the changes observed from the apical to the more basal sections is provided in figure 4:2:6 and 4:2:8.

To examine the protein composition of different tissues in the pea root, 1mm serial sections were dissected into two zones as described in section 2 A, and electrophoresed as described in chapter 2 section 2 G. Proteins from these small zones were silver stained to permit detection of very small amounts of protein. Results are summarised in figure 4:2:6. Considerable differences were observed both qualitatively and quantitatively between tissues as well as between apical and more mature zones. Differences between tissues were evident at the earliest stage examined, 1-2mm from the apex. Several tissue-specific proteins were observed, and development of the pattern of protein composition was characteristic of each tissue. There was no indication that individual proteins all underwent the same course of development in all tissues at the same transverse level, as certain proteins decreased while rising or remaining constant in other tissues, and as some proteins altered at varying distances from the apex depending on the tissue.

Figure 4:2:6. Developmental changes in protein from pea root tissue as analysed by two-dimensional polyacrylamide gel electrophoresis

Serial 300μ or one millimetre sections of pea root were dissected into cortex and stele, or into epidermis and stele + cortex. The sections were then homogenised in buffer containing 9.5M urea. Polypeptides were separated by NEPHGE in the 1st dimension and by SDS-PAGE in the 2nd dimension as described in chapter 2 section 2 G iii. Proteins were then silver stained essentially according to Switzer *et al.* (1979) as described in chapter 2 section 2 G iv b.

a) Two-dimensional electrophoresis of polypeptides obtained from cortical tissue 1-2mm from the apex.

b) Two-dimensional electrophoresis of polypeptides obtained from stelar tissue 1-2mm from the apex.

Many polypeptides were observed to differ in amount and/or pattern of alteration between the cortex, stele, epidermis and root cap. Some of these are indicated in figures 4 a and b as follows:

↙ Polypeptides higher in the stele than the cortex
↗ Polypeptides lower in the stele than the cortex

A minimum of 110 polypeptides were observed to differ qualitatively or quantitatively between the stele and the cortex in the zone 1-2mm from the apex. A predominance of high molecular weight and acidic polypeptides were observed in the stele, while low molecular weight and alkaline polypeptides were more prominent in the cortex.

Several polypeptides were examined in further detail, and these are described below.

1 This polypeptide is most prominent 700μ-2mm from the apex. It declines first in the epidermis, then cortex, and lastly the stele. It remains prominent in the stele 1-2mm from the apex. Except for a transient increase in the cortex 3-4mm from the apex, it remains low in amount after 2-3mm from the apex.

2 Relatively low in epidermal tissue throughout the entire apical 30mm. Prominent in other tissues 1-4mm from the apex, and declines in these after 4mm.

3 Most prominent in the stele, particularly 1-3mm from the tip. It is less prominent in the cortex, and very low in the epidermis. It declines in all tissues after 4mm.

4 This polypeptide remains lower in the stele than in the cortex throughout the entire 30mm.

5 These two polypeptides are in high concentration after 700μ, particularly in the stele. They are less prominent in the cortex, and relatively very low in the epidermis. After 5mm from the apex, they decline rapidly in the epidermis, more slowly in the cortex, and decline only slightly in the stele.

6 This polypeptide is prominent in the stele throughout the apical 30mm. It decreases in the epidermis after 4mm from the apex, and eventually becomes very low in both epidermis and cortex.

7 These four polypeptides are very high in amount 1-2mm from the apex, particularly in the stele where they remain high throughout the entire 30mm examined. After 2mm from the apex, they decline in the cortex and epidermis; the two lower molecular weight polypeptides declining more rapidly.

8 These two polypeptides increase rapidly to become prominent 1-2mm from the apex, particularly in the stele. This is followed by a rapid decrease to become very low in amount by 3mm from the apex in the epidermis, by 4mm in the cortex, and by 6mm in the stele.

9 and 10 These polypeptides were observed in the stele 1-2mm from the apex, and declined rapidly after 2mm. They were first observed about 2mm from the apex in cortical tissue and increased thereafter, to become prominent by 3-4mm from the apex.

15 Present only in the epidermis, after 19-20mm from the apex.

Fig. 4:2:6.

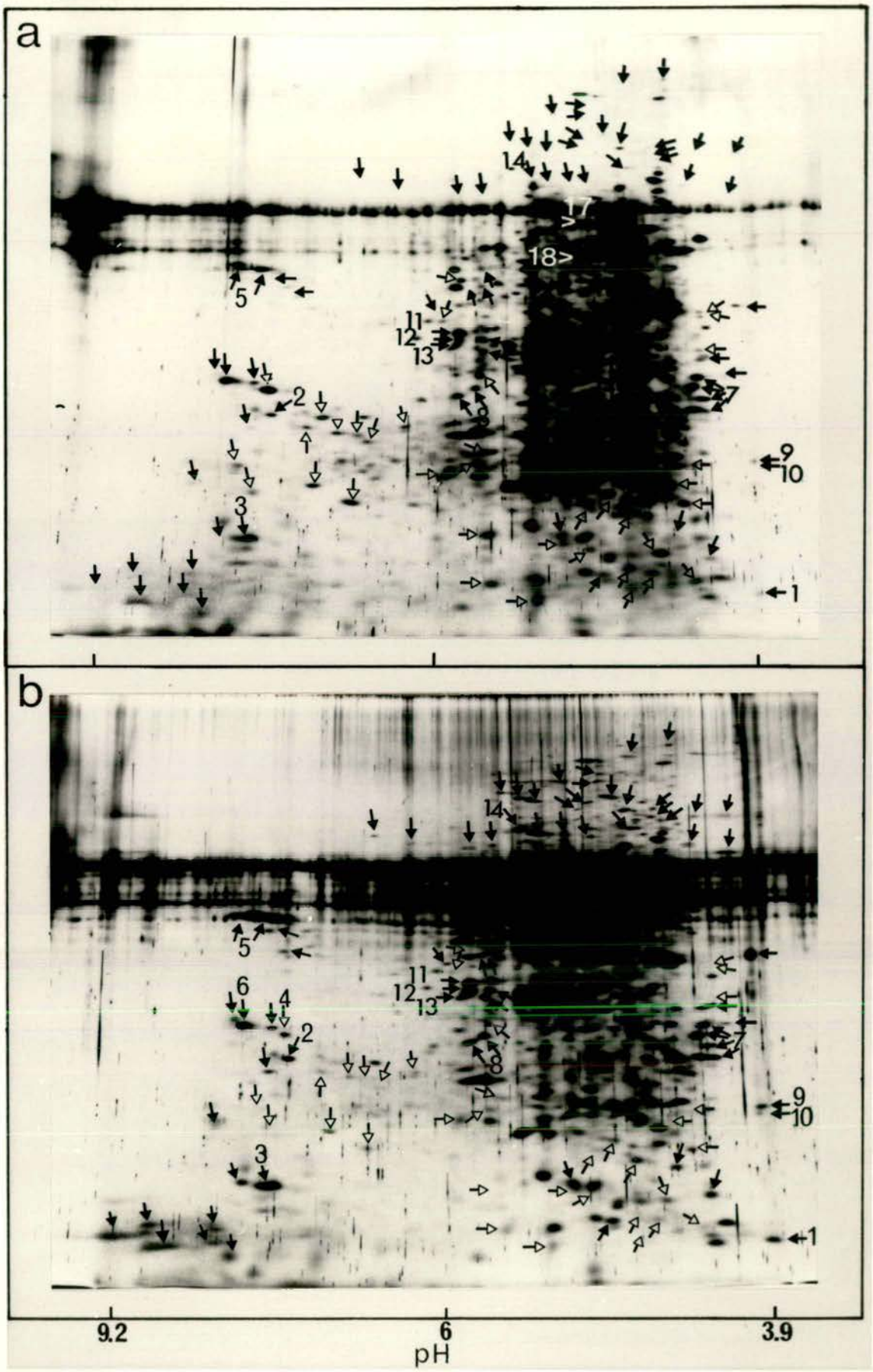


Fig. 4:1:19.

Figure 4:2:7. Fluorographs of (^{35}S) methionine labelled protein from pea root tissue separated by two-dimensional polyacrylamide gel electrophoresis employing NEPHGE in the first dimension

Pea roots were incubated for two hours in (^{35}S) methionine as described in chapter 2 section 2 F i. Successive one millimetre sections were dissected into stele and cortex + epidermis or into epidermis and stele + cortex, and homogenised in buffer containing 9.5M urea. Proteins were then separated in the first dimension by NEPHGE, and in the second dimension by SDS-PAGE as described in chapter 2 section 2 G iii as for figure 4:2:6. Radioactively labelled proteins were then detected by fluorography.

a) Fluorograph of proteins obtained from cortical tissue 3-4mm from the root apex.

b) Fluorograph of proteins obtained from stelar tissue 3-4mm from the root apex.

(i) Stage-dependent changes in the pattern of (^{35}S) methionine labelled proteins

Extensive changes in the pattern of *in vivo* labelling of proteins occurred with distance from the apex. Some of these are listed below.

1 Relatively high in amount 700µ-2mm from the apex. Declines after 3-4mm.
2 Increases rapidly in amount after 700µ from the apex and remains high until 4mm. Declines thereafter.
3 Very prominent in the apical mm. Declines after 4mm.
5 Declines after 6mm.
6 Prominent in the apical 2mm. Declines slowly thereafter.
7 These four polypeptides increase throughout the apical millimetre to become very prominent 1-2mm from the apex and decline thereafter.
8 These polypeptides, which are fairly low in the apical 3mm, are not detected after 3mm.
9 Present after 1mm from the apex.
10 Present after 1mm from the apex.
15 Present after 19mm from the apex.
16 Present after 19mm from the apex.

(ii) Tissue-dependent changes in the pattern of (^{35}S) methionine labelled proteins

The pattern of *in vivo* labelled proteins varied considerably between tissues as well as with distance from the apex. Some of these changes are indicated as follows:

↙ Higher in the stele than the cortex
↗ Lower in the stele than the cortex.

Approximately 120 quantitative and qualitative changes were observed between the cortex and stele in the zone 3-4mm from the root apex. Many of the polypeptides predominantly observed in the stele were high molecular weight and acidic, while a predominance of lower molecular weight and more alkaline or neutral polypeptides were associated with the cortex.

Several polypeptides were examined in further detail, and these are described below.

Polypeptides 1-10 followed a similar pattern of labelling to that of staining as described in figure 4:2:6.

Several polypeptides did not show a similar pattern of labelling to staining, including the following.

11 This polypeptide is present in the same amount in the cortex and stele throughout the entire 30mm. However, labelling declines beyond 1mm from the apex, then increases again, first in the stele 2-3mm from the apex, then in the cortex 5-6mm from the apex.

12 This polypeptide is present in the same amount in the cortex and stele throughout the entire 30mm. However, labelling declines beyond 1mm from the apex, then increases again, first in the stele 2-3mm from the apex, then in the cortex 5-6mm from the apex.

13 This polypeptide is present in the same amount in the cortex and stele throughout the entire 30mm. However, labelling declines beyond 1mm from the apex, then increases again, first in the stele 2-3mm from the apex, then in the cortex 5-6mm from the apex.

15 This polypeptide is not observed on stained gels until 19-20mm from the apex, and is present in the epidermis only. It becomes labelled in both the cortex and epidermis by 2-3mm from the apex, and is prominent after 5mm from the apex.

16 This polypeptide is not observed on stained gels until 19-20mm from the apex, and is present in the epidermis only. It becomes labelled in both the cortex and epidermis by 2-3mm from the apex, and is prominent after 5mm from the apex.

17 This polypeptide is present in high concentration in gels of both stele and cortex throughout the apex. However, although the labelling remains high in stelar tissue, it decreases rapidly in the cortex after 2-3mm from the apex.

Fig 4:2:7.

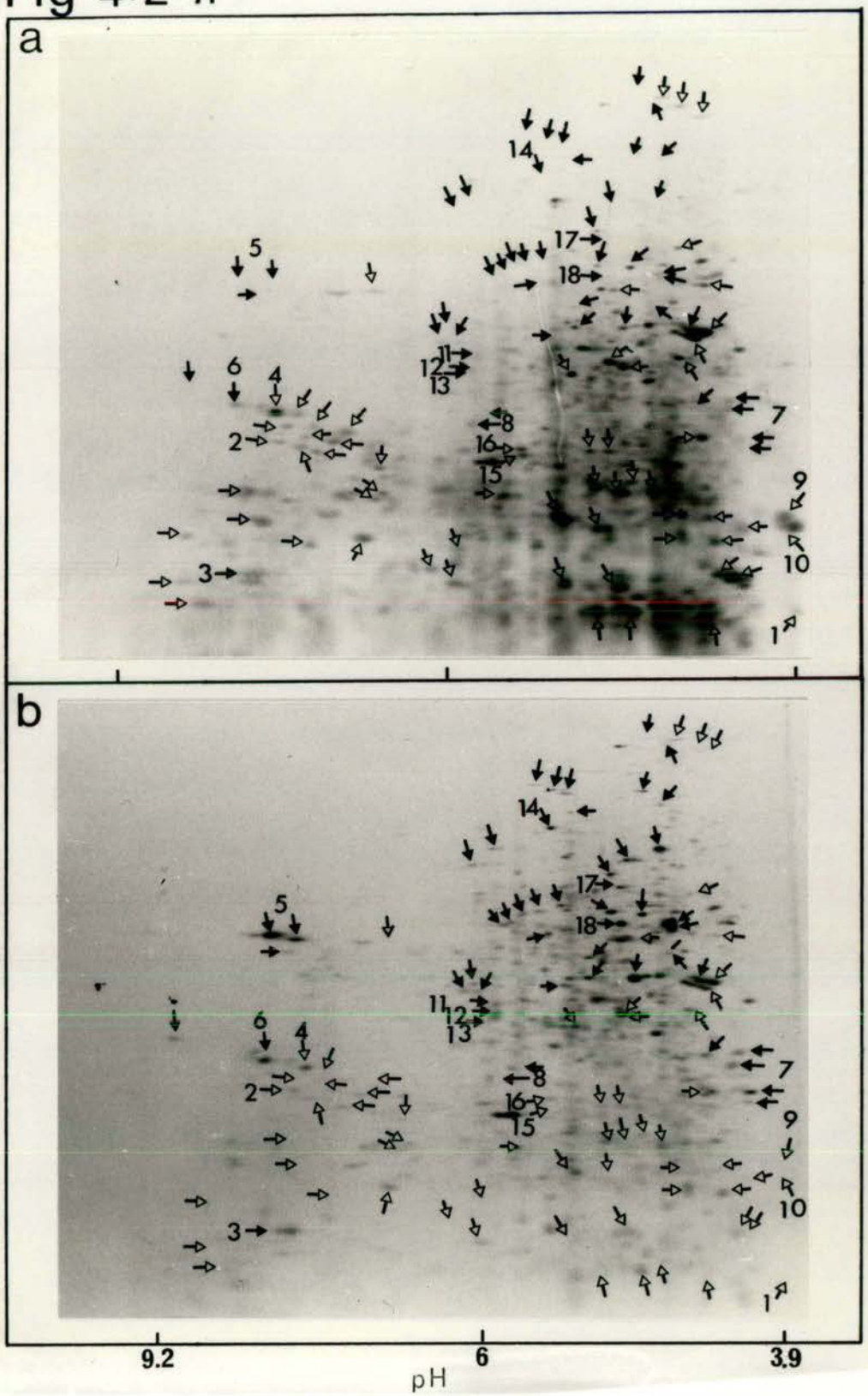


Figure 4:2:8. Autoradiographs of (^{35}S) methionine labelled proteins from pea root tissue separated by two-dimensional polyacrylamide gel electrophoresis employing isoelectric focusing in the first dimension

Pea roots were incubated for two hours in (^{35}S) methionine as described in chapter 2 section 2 F i. Successive 300µ, 400µ, or one millimetre sections over the apical 30 millimetres were cut, and were either left intact, or further dissected into cortex and stele. The tissue was then homogenised in buffer containing 9.5M urea. Proteins were then separated in the first dimension by isoelectric focusing, and in the second dimension by SDS-PAGE as described in chapter 2 section 2 G iii. Radioactively labelled proteins were subsequently detected by autoradiography.

a) Autoradiograph of proteins obtained from root cap tissue 0-300µ from the root apex

b) Autoradiograph of proteins obtained from tissue 700-1000µ from the root apex

c) Autoradiograph of proteins extracted from tissue 5-6mm from the root apex

(i) Stage-dependent changes in the pattern of (^{35}S) methionine labelled proteins

Extensive changes occurred in the pattern of labelled proteins during development in the apical 20mm of the pea root apex. Some of these are indicated in figures a-c as follows:

- ➤ Polypeptides increase with distance from the apex either with respect to the apical 300µ, or to the section 700-1000µ from the root apex.
- ⊀ Polypeptides decrease with distance from the apex either with respect to the apical 300µ, or to the section 700-1000µ from the root apex.

Approximately 165 quantitative and qualitative changes in polypeptides were readily detectable between the apical millimetre and the zone 5-6mm from the apex as shown in figure 4:2:8.
Several proteins were examined in greater detail, and the changes occurring with distance from the apex are described below.

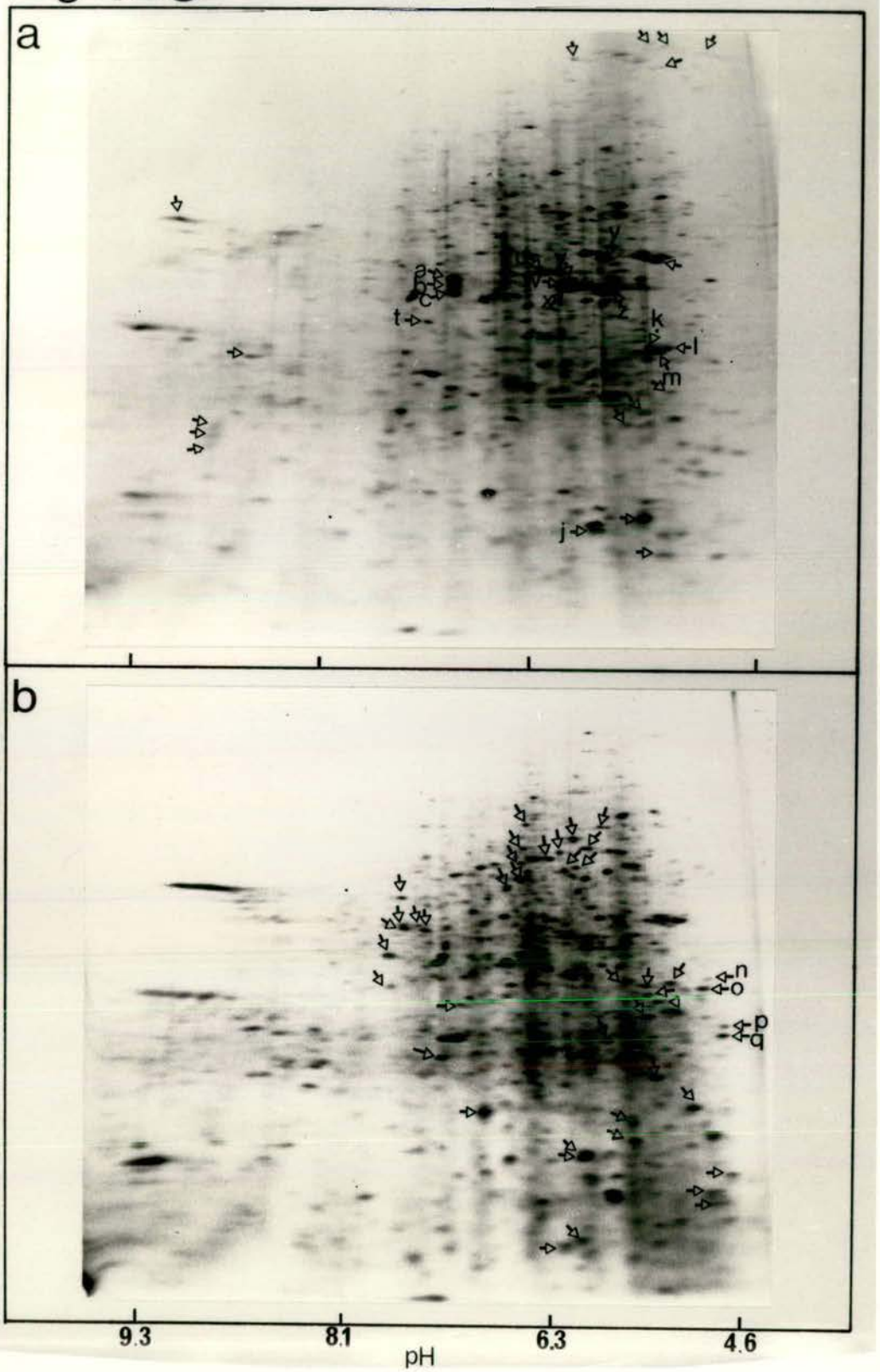
a. Present only in the apical 300µ
b. Present only in the apical 300µ
c. Present only in the apical 300µ
d. Present only after 2-3mm from the apex
e. Present only after 2-3mm from the apex
f. Present only after 2-3mm from the apex
g. Present only after 2-3mm from the apex
h. Present only after 2-3mm from the apex
i. Present after 700µ from the apex
j. Absent after 3mm from the apex
n. Declines rapidly after 2-3mm from the apex
o. Declines after 2-3mm from the apex
p. Declines rapidly after 2-3mm from the apex
q. Declines rapidly after 2-3mm from the apex
r. Present after 1mm from the apex
s. Present after 1mm from the apex

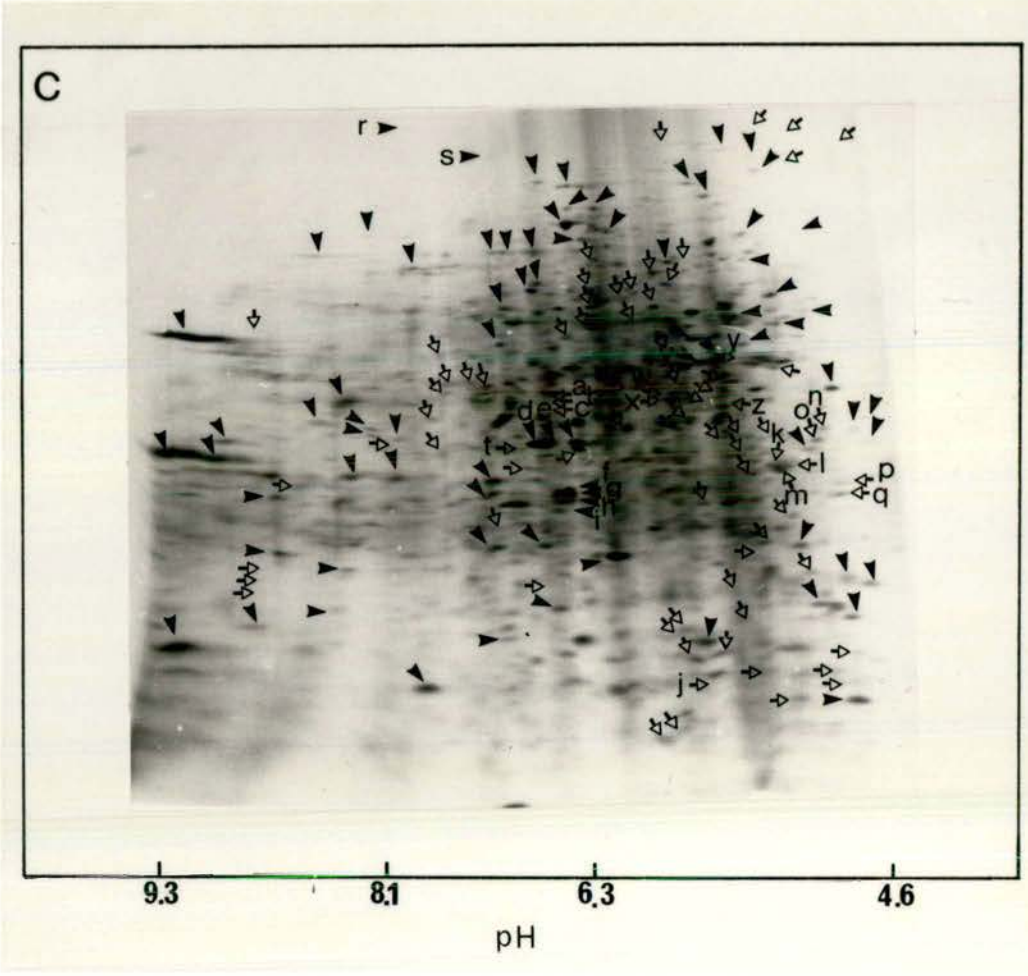
(ii) Tissue-dependent changes in the pattern of (^{35}S) methionine labelled proteins

Labelling of many polypeptides altered considerably between tissues as well as with distance from the apex throughout differentiation in the apical 30mm of pea root. Changes in several of the polypeptides examined in detail are outlined below.

a. Present only in the apical 300µ. (Unique to root cap)
b. Present only in the apical 300µ. (Unique to root cap)
c. Present only in the apical 300µ. (Unique to root cap)
d. Not detected in the root cap. Detected after 2-3mm from the apex, remaining low in the stele although prominent in the cortex
e. Not detected in the root cap. Detected after 2-3mm from the apex, remaining low in the stele although prominent in the cortex
f. Not observed in root cap or stelar tissue. Present in the cortex after 2-3mm from the apex
g. Not observed in root cap or stelar tissue. Present in the cortex after 2-3mm from the apex
h. Not observed in root cap or stelar tissue. Present in the cortex after 2-3mm from the apex
i. Not detected in the root cap. Detectable after 1mm, increasing in the cortex although remaining low in the stele.
j. Prominent in the root cap and stele, remaining low in the cortex
k. Prominent in the root cap and cortex, remaining low in the stele
l. Prominent in the root cap and stele, remaining low in the cortex
m. Prominent in the apical millimetre, remaining very prominent in the stele
n. Declines in cortex after 2-3mm
o. Prominent in the apical millimetre, remaining very prominent in the stele, while declining in the cortex after 2-3mm
p. Declines rapidly in the cortex after 2-3mm
q. Declines rapidly in the cortex after 2-3mm
r. Not detected in root cap or stele. Increases in the cortex after 1mm
s. Not detected in root cap or stele. Increases in the cortex after 1mm.
t. Present only in the apical 300µ. (Unique to the root cap)
u. Very prominent in the root cap
v. Very prominent in the root cap
w. Very prominent in the root cap
x. Very prominent in the root cap
y. Very prominent in the root cap
z. Very prominent in the root cap

Fig. 4.2.8.




C
r
s
9.3
8.1
6.3
4.6
pH

Differentiation therefore appeared to be accompanied by substantial differences in protein composition that could be detected from very early stages of differentiation. As the minimal variations observed in mRNA species could not account for the extent of changes in protein composition, the control of protein synthesis was further investigated by observing the pattern of *in vivo* labelled proteins by autoradiography. The results of autoradiography of proteins separated by isoelectric focusing in the first dimension are summarised in figure 4:2:8 and by non-equilibrium pH gradient electrophoresis in figure 4:2:7. Differences observed with increasing distance from the apex are outlined in figure 4:2:7i and 4:2:8i; whereas some of the differences associated with tissues are outlined in figure 4:2:7ii and 4:2:8ii.

Again considerable differences were observed in the pattern of *in vivo* labelled protein between the apical and more mature zones, and between different tissues, using a variety of extraction methods including SDS, 9.5M urea, sonication in RNase or SDS as described in chapter 2 section 2 G iii. Results from isoelectric focusing and from NEPHGE urea-extracted proteins only are outlined in figure 4:2:7 and 4:2:8. As observed for protein composition, the differences were far more extensive than those observed in *in vitro* translation products.

In order to obtain an indication of whether the control of protein synthesis might therefore be largely at the level of translation, polypeptides of the same pI' and molecular weight values were compared. The comparison showed that many of the changes occurring in proteins during differentiation had a corresponding change in apparent rate of protein synthesis as estimated by autoradiography of *in vivo* labelled proteins, thus the control of at least some proteins was probably at the level of translation. However, some proteins did not show such a parallel change, including the polypeptides 11-13, 15-17.

Polypeptide 17, for example, is observed in high concentration in gels of the stele and cortex throughout at least the apical 6mm. However, although remaining high in autoradiographs of the stele, it decreased rapidly in the cortex, and after 3mm was hardly detectable in autoradiographs of cortical tissue. This

differential rate of synthesis in conjunction with similar protein concentration implies a differential rate of breakdown of the protein between the stele and cortex beyond two millimetres from the root apex. For example, there may be a much lower rate of breakdown in the cortex as well as a decreased rate of synthesis so that the protein accumulates without concurrent protein synthesis.

Similarly, polypeptides 11-13 remain in stained gels at the same concentration in the cortex and stele throughout the apical 20mm. However, although high in the apical millimetre on autoradiographs, their synthesis decreases thereafter until increasing again 2-3mm from the tip in stelar tissue and 5-6mm in the cortex. The apparently lower rate of synthesis and later increase in rate of synthesis in the cortex than in the stele although the protein concentration remains similar throughout development, indicates again that there may be differential rates of breakdown of protein as well as differential rates of synthesis.

Polypeptides 15 and 16 are prominent in the cortex and epidermis in autoradiographs after 2-3mm from the root apex. However, they are not observed on stained gels until 19-20mm from the apex, being detected only on gels of epidermal tissue. This again indicates differential breakdown of the protein, or post-translational modification in different zones as well as between different tissues.

It should also be noted that alteration of individual proteins did not necessarily coincide in different tissues. For example polypeptides 9 and 10 were observed in the stele from 1-2mm from the apex in both gels and autoradiographs. However, there was a rapid decrease thereafter, and they were not detected after 2mm from the apex in the stele. By contrast, they appeared in the cortex only after 3.5mm when they became very prominent in both gels and autoradiographs. Similarly, synthesis of polypeptides 11-14 was initially observed in the stele 1-2mm (14) or after 2mm (11-13), whereas synthesis did not increase or become detectable until after 5-6mm in the cortex. Other polypeptides which were only observed in some tissues were either present throughout differentiation, or appeared at

a specific stage of differentiation (for example polypeptides D-I).

In these preliminary generalised observations on protein composition evidence was obtained that protein composition changes considerably throughout differentiation in the pea root. As *in vitro* translatable mRNA altered very little during differentiation, most of the change in protein composition could not be accounted for by control at the level of transcription. It appeared that post-translational and post-transcriptional/translational control mechanisms may be involved in regulation of protein composition. The further analysis of protein and *in vitro* translation products therefore extends the earlier observation (section 2 B iv) that as protein synthesis/cell decreases rapidly while rRNA and *in vitro* translatable mRNA both increase/cell that some form of post-transcriptional control of protein synthesis is probably operating, as it appears from two-dimensional protein analysis that both qualitative and selectively quantitative post-transcriptional controls are operating.

Although the results were consistent with the observation of Brown (1963) in that differentiation is associated with alterations in relative enzyme activities (Brown equated enzyme activity with protein content in the root apex), the results were not in agreement with the hypothesis of the manner in which enzyme activities or protein content altered during differentiation. The results differed in several major respects.

1. Tissue-specific proteins were detected
2. Stage-specific proteins were detected
3. The rise and/or fall in concentration of individual proteins did not always coincide at the same transverse level in different tissues
4. Differences in protein composition between tissues were not limited to differences in the rate or extent of changes in individual proteins
5. Change in concentration of individual proteins was not always accompanied by a corresponding change in their synthesis
6. No evidence was obtained for transcriptional control as a major control point in differentiation.

The implications of these observations will be discussed in chapter 5.

Although two-dimensional polyacrylamide gels of crude extracts of pea root indicated the extent of change in protein composition, the results are open to many objections particularly on the grounds of differential extractability or modification of individual proteins between different zones; accessibility of labelled amino acids; the validity of comparison of polypeptides simply on the basis of molecular weight and pI'; lack of knowledge of function and activity of the polypeptides concerned.

Furthermore, *in vivo* and *in vitro* translation products could not be matched without further identification. This was partly due to the uncertainty that the pI' and molecular weight of the *in vivo* and *in vitro* products were identical; thus, for example, polypeptides migrating to the same position on two-dimensional gels of both *in vivo* and *in vitro* products might in fact be different proteins, or alternatively, the same protein might migrate to a different position in the gels. A different pI' and molecular weight could arise as a result of lack of co- or post-translational processing of *in vitro* translation products, or to reading of incompletely processed transcripts, or to misreading of the message. Additional means of identification of individual proteins are therefore necessary for comparison of protein synthesised *in vivo* and *in vitro*.

Due to problems of interpretation of apparent changes in protein or mRNA levels and their significance, it was therefore thought more useful to compare the level, activity and synthesis of individual proteins.

(iii) Developmental changes in individual proteins

a) Introduction

Individual proteins were studied in greater detail in order to examine more reliably the control of protein concentration and to investigate the function of some of the changes in protein levels during differentiation. In the context of Brown's model (1963) of root differentiation as described in chapter 1, it was thought appropriate to choose a regulatory

non-enzyme protein that was potentially involved in differentiation; and to investigate not only the concentration, synthesis and mRNA abundance of this protein, but also its regulation of enzyme activities, and its potential function during differentiation. This was to investigate several aspects of the model: 1. Whether enzymes varying in amount may have a functional role in differentiation. 2. If the above is found to be the case, whether the pattern of variation occurs in the manner specified by the model. 3. Whether a non-enzyme regulatory protein might be involved in differentiation. 4. Whether a non-enzyme protein involved in differentiation varies in amount during differentiation. 5. To investigate further pre- and post-translational regulation of protein concentration and activity during differentiation.

One of the proteins appearing to vary during differentiation as analysed by gel electrophoresis had similar molecular weight and pI' values to calmodulin (protein 1 figures 4:2:6, 4:2:7 and 3:2:8). As described in chapter 1 section 1 calmodulin is a major regulatory protein acting on proteins and enzymes known to be important in processes involved in plant differentiation that vary throughout root development. These include tubulin aggregation, organisation of the spindle during mitosis, geotropism, and NAD kinase activity. Furthermore, it is known to be regulated post-translationally by calcium ion concentration, and additionally to regulate both directly and indirectly the level and distribution of this crucial regulatory ion. I therefore attempted to identify and characterise this protein further in order to obtain a more reliable and meaningful estimate of the concentration, synthesis and mRNA abundance of one protein. Preliminary investigations of its potential function during differentiation were also carried out.

b) Developmental changes in calmodulin

As described in chapter 3 section 2, assay of calmodulin involves a number of problems, particularly in relation to extractability, and to interference by calmodulin-binding proteins and calmodulin-like proteins. Attempts to overcome these problems are described in chapter 3 section 2. As it is desirable to estimate calmodulin by more than one method, I

assayed this protein by biological activity as well as by presence on two-dimensional polyacrylamide gels.

I. Analysis of calmodulin during differentiation by two-dimensional polyacrylamide gel electrophoresis

In order to minimise problems of interference and identification, I attempted to purify calmodulin prior to assay. As described in chapter 3 section B ii e and B iii b, a protein possessing many of the characteristics of calmodulin was purified almost to homogeneity as analysed by two-dimensional polyacrylamide gel electrophoresis. However, no polypeptide could be detected on gels that had the electrophoretic characteristics of calmodulin. This might have been the result of modification of the protein (chapter 3 section 2 B iii b) altering its electrophoretic characteristics (for example Burgess *et al.*, 1980), or of limited proteolysis as has been noted in isolation of calmodulin from other species (Charbonneau *et al.*, 1980, Esnouf *et al.*, 1980). The estimation of purified calmodulin on two-dimensional polyacrylamide gels could therefore not be carried out.

In crude homogenates, the polypeptide of similar molecular weight and pI' to calmodulin was, however, observed to undergo a calcium-dependent electrophoretic mobility shift characteristic of calmodulin (chapter 3 section 2 A iii b and c; 2 C ii). Attempts to identify calmodulin further on polyacrylamide gels by affinity labelling after Western transfer with radioactively labelled phenothiazines were only partially successful, and calmodulin could therefore not be identified more reliably. However, as the nature and extent of the calcium-dependent mobility shift is so distinctive of calmodulin it appears that it may be used to distinguish calmodulin from other calcium-binding proteins and calmodulin-like proteins (chapter 3 section 2A iii b). Furthermore, no other polypeptide migrated to a similar position on two-dimensional polyacrylamide gels, and no other polypeptide could be detected to undergo a calcium-dependent mobility shift. This protein was therefore tentatively identified as calmodulin on two-dimensional polyacrylamide gels of pea root homogenates, and will be referred to as calmodulin. Differential extractability

or modification of calmodulin during extraction and electrophoresis, and interference by calmodulin-binding proteins did not appear to be a problem under the conditions employed as described in chapter 3 section 2 C ii b.

As described in figure 4:2:6, calmodulin was found to alter both in concentration and in relation to other proteins during differentiation as analysed by two-dimensional polyacrylamide gel electrophoresis. Protein content was too low to allow detection of calmodulin in the apical 700μ; however, calmodulin was in highest amount/section 700-1000μ from the apex, decreasing fairly rapidly thereafter first in the epidermis, then the cortex, and then the stele. The concentration in stelar tissue 1-2mm from the apex therefore appeared to be higher than in other tissues. Calmodulin reached a minimum in all tissues by 2-3mm from the apex. There was a very slight increase in concentration in the cortical section 3-4mm from the apex before decreasing again.

The quantitative changes occurring in this protein were found to be paralleled by changes in the degree of *in vivo* labelling indicating that change in its concentration reflected the degree of its concurrent synthesis. As so few *in vitro* translation products appeared to alter during development of the root, it was therefore of great interest to investigate whether *in vitro* translation of calmodulin altered.

A polypeptide from *in vitro* translation products which was found to migrate to an identical position in two-dimensional polyacrylamide gels to calmodulin in crude homogenates, underwent an identical calcium-dependent electrophoretic mobility shift as described by Van Eldik *et al.* (1980a) for calmodulin translated *in vitro* from spinach mRNA. Unfortunately, being such an acidic protein, and therefore on the limit of detection of the pH range of NEPHGE gels and not observed on isoelectric focusing gels, it was not observed on all gels of *in vitro* translation products. Due to lack of time, the experiments could not be repeated and results of the relative abundance of the mRNA for the calmodulin-like protein during development are inconclusive.

I attempted to isolate calmodulin from *in vitro* translation

products by phenothiazine affinity chromatography as described in chapter 2 section 2 J vi and 2 H i c II. As described in chapter 3 section 4 C ii, wheatgerm calmodulin and TCA-precipitable radioactivity bound to the affinity column in a calcium-dependent manner and it therefore seemed probable that calmodulin or a calmodulin-like protein was present in *in vitro* translation products of pea root mRNA. Unfortunately, no polypeptide was detected on two-dimensional gels of the EGTA eluate, probably due to limited proteolysis or modification of calmodulin during affinity chromatography as described in chapter 3 section 2 B iii b. Quantitation of calmodulin in *in vitro* translation products by affinity chromatography was therefore not attempted.

II. Estimation of calmodulin by biological assay

In order to both estimate the concentration of calmodulin during differentiation using a different technique, and to quantitate the protein, calmodulin was extracted from serial sections and assayed by its ability to activate NAD kinase.

A calmodulin-like protein was purified almost to homogeneity as described in chapter 2 section 2 H i c I and chapter 3 section B ii, both to minimise interference in its assay particularly by calmodulin-binding proteins and calmodulin-like proteins, and to solubilise calmodulin from membranes and organelles. Although not demonstrated conclusively to be calmodulin it possessed many of the characteristics of calmodulin, including heat stability, acidic isoelectric point, ability to activate calmodulin-dependent isozymes of bovine brain phosphodiesterase and plant NAD kinase, calcium-dependence of enzyme activation, susceptibility of activation to low concentrations of phenothiazines, and ability of a component of this fraction with calmodulin-like properties to bind to a phenothiazine affinity column. The highly-purified protein was then assayed by its ability to activate calmodulin-dependent calmodulin-deficient NAD kinase extracted by the method of Anderson *et al.* (1980) (chapter 2 section 2 H ii a) and assayed by a modification of the method of Muto and Miyachi (1977) as described in chapter 2

section 2 I ii and iii, and chapter 3 section 3 B ii. Results are shown in figure 4:2:9.

The estimates of calmodulin/section from NAD kinase assay corresponded to the relative amounts detected by two-dimensional polyacrylamide gel electrophoresis. The concentration of extractable calmodulin (estimated on the assumption that 1mm^3 of tissue was equivalent to 1µl, and a molecular weight estimate of 17,000 daltons for calmodulin) was found to be extremely high in the apical 1.2mm, particularly in the root cap including the root cap columella. (As extractable calmodulin was less than 100ng in the root cap in the apical 300µ, however, it was not detected on two-dimensional polyacrylamide gels of the root cap). There was a very sharp drop in extractable concentration from an estimated 170µM in the root cap where it represented almost 1% of soluble protein to 70µM in the region of the quiescent centre and proximal 300µ of the apical meristem where it constituted 0.23% of soluble protein, to a minimum of 10µM immediately beyond the apical meristem where it constituted only 0.04% of soluble protein. There appeared to be a brief period during which calmodulin was at a minimum concentration between 1.2-1.6mm from the apex, before rising to a second, smaller peak between 2.5-4mm where it rose to 22µM and was estimated to constitute 0.23% of soluble protein. The concentration thereafter fell steadily to 10mm beyond the apex to about 10µM, and remained at about 0.35% of soluble protein after 4.5mm from the apex.

It appeared from two-dimensional polyacrylamide gel electrophoresis that the concentration of calmodulin in the apex of the root proper declined first in the epidermis, then in the cortex, and lastly in the stele, reaching a minimum in all tissues 2-3mm from the apex. The small second peak appeared to occur in the cortex between 2.5-4mm from the apex.

It was noted that of those tissues of the root proper isolated for estimation of calmodulin by two-dimensional polyacrylamide gel electrophoresis, the epidermis in the apical meristem was the first to cease division, whereas divisions could be observed in stelar cells for longer than in adjacent cortical

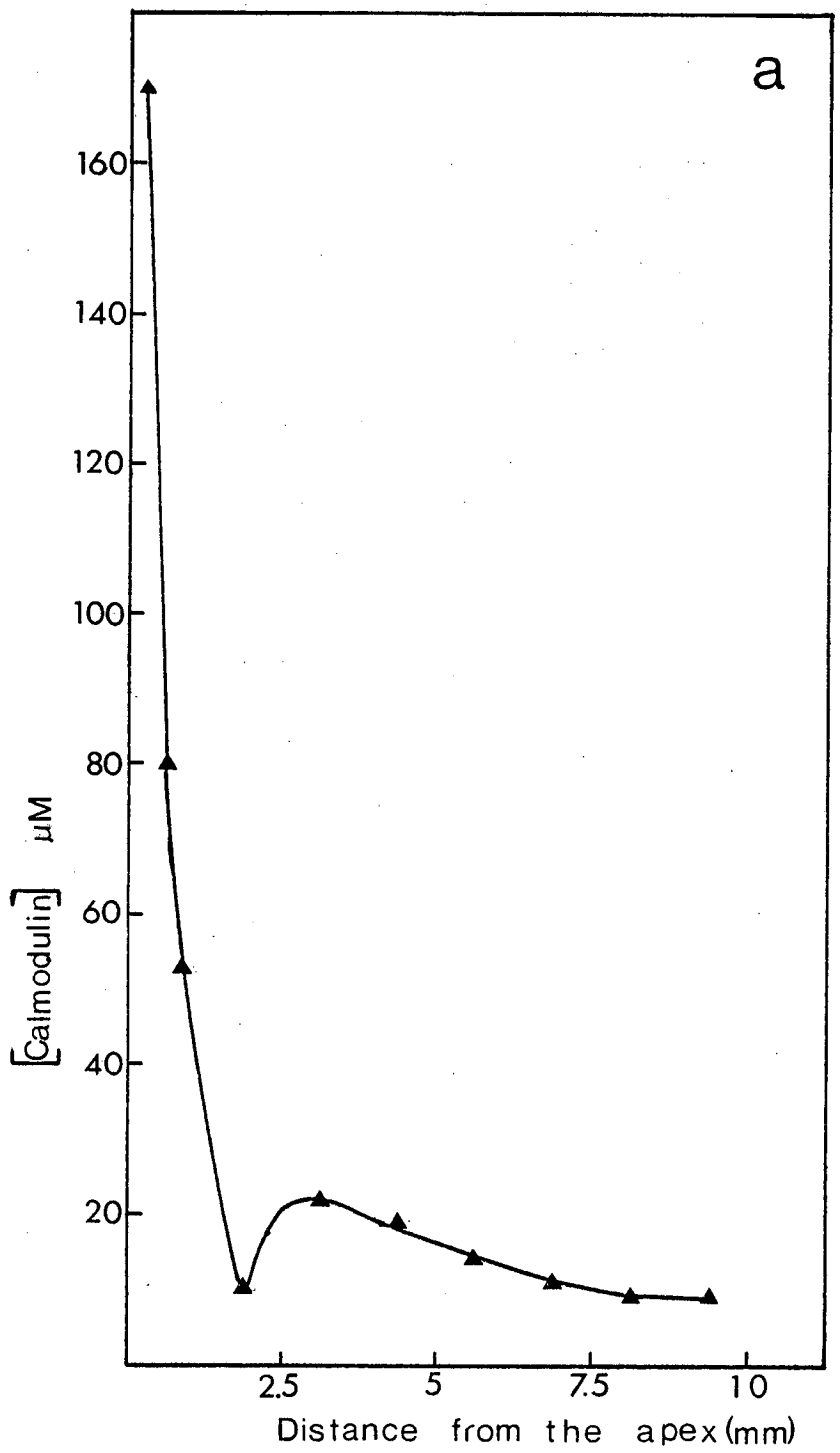
Figure 4:2:9. Developmental changes in calmodulin in the root apex

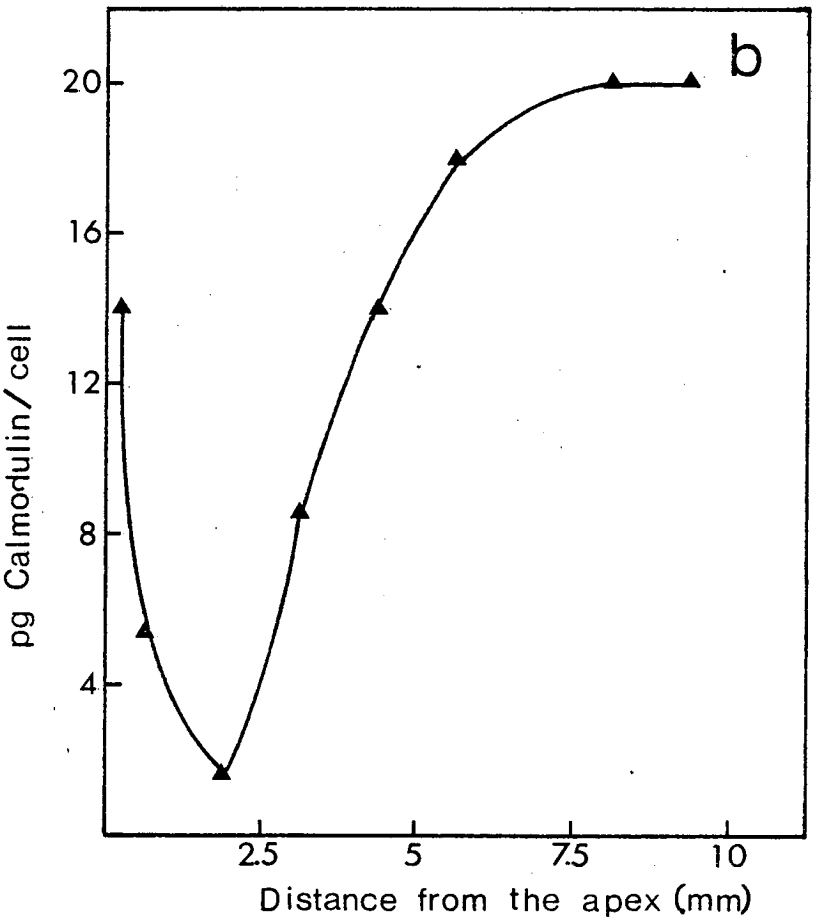
Calmodulin was extracted from serial 1.25mm sections of pea root as described in chapter 2 section 2 B ii a, and assayed by its ability to activate calmodulin-dependent, calmodulin-deficient NAD kinase by the modified procedure of Muto and Miyachi (1977) as described in chapter 2 section 2 I iii. The concentration of calmodulin was then estimated by comparison with the activating ability of known amounts of bovine brain calmodulin.

a) Developmental changes in the concentration of calmodulin.

b) Developmental changes in the content of calmodulin per cell.

Fig. 4:2:9.


a
160
140
120
100
80
60
40
20
[Calmodulin] µM
2.5
5
7.5
10
Distance from the apex (mm)


b
20
16
12
8
4
pg Calmodulin / cell
2.5
5
7.5
10
Distance from the apex (mm)

and epidermal cells. The decrease in calmodulin therefore closely followed the tissue distribution of cessation of division in the apical meristem. The second peak in calmodulin, which occurred in the cortex, also coincided with the zone of frequent cell division in inner cortical, endodermal and pericycle cells opposite protoxylem arcs in the meristems of lateral root primordia. The zones of relatively high calmodulin concentration therefore coincided closely with areas of high frequency of cell division, and perhaps in the case of calmodulin, more pertinently, in the zones of high mitotic frequency.

The amount of extractable calmodulin/cell was also found to be high in the root cap at 14pg, and, as for concentration and % of soluble protein, decreased rapidly to a minimum 1-2mm beyond the apex at 1.5pg/cell. By 2.5mm it began to rise rapidly to a maximum of 20pg/cell about 8mm beyond the apex.

This pattern in concentration of calmodulin was consistently obtained. As described in chapter 3 section 2 B ii, efforts were made to avoid problems of differential extractability due to binding to calmodulin-binding proteins, membranes or organelles. No evidence was obtained for significant interference by activators or inhibitors of either NAD kinase or calmodulin in different sections, while the extraction procedure did not appear to alter activating ability (chapter 3 section 2 B iii; C i, and 3 B ii c). As the results also correlated well with those from two-dimensional polyacrylamide gel electrophoresis using a totally different extraction procedure as well as assay method, it is probable that the trend in relative concentration of calmodulin during development resembles the curve observed by biological activity.

It should be noted that the actual concentration of calmodulin may be quite different from the values indicated, not only as despite rigorous extraction conditions a certain proportion of calmodulin may not have been extracted, but also as estimates of calmodulin based on activating ability were obtained relative to the activating ability of bovine brain calmodulin which may have a lower activating ability for NAD kinase than pea calmodulin (Cormier *et al.*, 1981, 1982). This would tend to overestimate the amount of calmodulin in pea roots.

However, it is also possible that the partially purified calmodulin samples were present in the form of fragments of lower specific activity than the entire calmodulin molecule, thus tending to underestimate the concentration of calmodulin considerably (Charbonneau *et al.*, 1980; Cormier *et al.*, 1981). The latter is perhaps less likely as activating ability of bovine brain calmodulin was not modified by extraction with pea calmodulin (chapter 3 section 2 B iii a).

It therefore appears that the concentration of calmodulin is relatively very high in the root cap and apical meristem, falling rapidly to a minimum immediately behind the apical meristem although remaining high in the later-dividing cells of the stele. There is a second, much smaller, peak in concentration 2.5-4mm beyond the root apex, probably occurring in the cortex, and coinciding with the period of more frequent cell division in the formation of lateral root primordia in the inner cortex, and of autophagy in cortical vacuoles. There was a slow decline in concentration thereafter. Estimates of calmodulin varied from a minimum of 10μM 1.2-1.6mm and beyond 8mm from the apex, to 170μM in the root cap. Calmodulin varied from 0.04% of total protein 1-2mm from the root tip to a maximum of about 1% in the root cap. Calmodulin/cell was again at a minimum of 1.5pg 1-2mm from the root tip and rose to a maximum of 20pg/cell by 8mm from the tip.

c) Developmental changes in calmodulin-binding proteins

I. Presence of calmodulin-binding proteins in the root apex of *Pisum sativum*

In view of the considerable and rapid variations in calmodulin during differentiation it was of interest to know whether calmodulin might have some function in differentiation and whether the concentration of calmodulin to any extent might regulate its function.

As mentioned above, areas of high concentration of calmodulin coincided with zones of high cell division and mitotic frequency. As calmodulin is known to be required in high concentration for control of polymerisation of microtubules and is known to have

a dynamic role in the spindle during mitosis, the high concentration of calmodulin might at least in part reflect the requirement for mitosis. However, it was of interest to investigate whether calmodulin-binding proteins other than tubulin were found in roots in order to investigate the potential range of functions of calmodulin.

I therefore attempted to purify calmodulin-binding proteins by calcium-calmodulin affinity chromatography as described in chapter 2 section H iii b and chapter 3 section 3 A iii, and the EGTA eluate was analysed by two-dimensional polyacrylamide gel electrophoresis and NAD kinase assay as described in chapter 2 section 2 I ii and chapter 3 section 3 A iii.

As shown in figure 3:2:10 several polypeptides bound to a calmodulin affinity column in a calcium-dependent manner. Four major, and twelve minor polypeptides were detected. No polypeptides were detected in the calcium eluate immediately prior to EGTA elution, therefore these polypeptides bound in a calcium-dependent manner to the column. In order to further investigate whether these polypeptides were specifically bound to calmodulin, the calmodulin-free, calmodulin-binding proteins NAD kinase and phosphodiesterase were electrophoresed on non-denaturing gels in the presence of calmodulin and either calcium or EGTA, as described in chapter 2 section 2 G ii. However, as the attempts were unsuccessful, the use of non-denaturing gels to demonstrate calmodulin-binding proteins from the calmodulin affinity column was not attempted. The specificity of binding could be further investigated by applying radioactively labelled calmodulin to Western transfers of polyacrylamide gels containing these proteins.

None of the proteins observed had the molecular weight of the calmodulin-dependent NAD kinase estimated to be 57,000 by Jarrett *et al.* (1983). As described in chapter 3 section 3 A iii some calmodulin-dependent NAD kinase activity did bind to the column in a calcium-dependent manner, but it is probable that the enzyme is so unstable that insufficient concentration was present by the time of elution from the affinity column to be detected on polyacrylamide gels. As it appeared that pea roots might contain several calmodulin-binding proteins, I therefore attempted to investigate one calmodulin-binding protein, NAD kinase, in greater detail.

II. Developmental changes in NAD kinase

Preliminary estimates of NAD kinase activity during differentiation were obtained as described in chapter 2 section 2 H ii c and I ii, and chapter 3 section 3 B ii c and C.

Two forms of NAD kinase were observed in pea shoot tissue. As described in chapter 3 section 3 A one of these was completely dependent on calmodulin and calcium for activity, and the other was completely independent of calmodulin and calcium. Extraction conditions in root tissue were therefore designed to extract both these forms, and estimates of both were obtained by the modified assay of Muto and Miyachi as described in chapter 2 section 2 I ii, and chapter 3 section B ii. Activators or inhibitors of the NAD kinase assay did not affect the results to any significant extent. Results obtained for NAD kinase activity during differentiation in the root apex are shown in figure 4:2:10.

Total NAD kinase activity when fully saturated with calmodulin was found to rise about 17-fold on a cell basis, 5-6-fold on a protein basis, and 3-fold on a section basis between the tip and 20mm from the tip. The increase between 5 and 20mm was therefore similar to the 3-4-fold increase in the NADP+NADP(H):NAD+NADH ratio and the 6-fold increase in NADP:NAD ratio occurring in *Vigna* roots over this zone (Yamamoto, 1963), indicating that the increase in ratio may be due to a concurrent increase in NAD kinase activity.

Most NAD kinase activity in shoot tissue is calmodulin-dependent and is present in chloroplasts (Muto *et al.*,1981; Jarrett *et al.*, 1982a) and is thought to be activated by a light (phytochrome)-induced influx of calcium to produce NADP for use in photosystem I. This function for NAD kinase is not applicable in roots. However, NADP and NADP-dependent enzymes are known to be present in roots and to vary throughout the root tip (for example Fowler and Ap Rees, 1970), being required in biosynthetic processes involved in differentiation. As NADP is thought to be a limiting factor in at least some NADP-requiring processes (Fowler and Ap Rees, 1970), NAD kinase activity might have a regulatory role in developmental processes. Furthermore, as NADP is found in both plastids and cytoplasm, and cannot cross

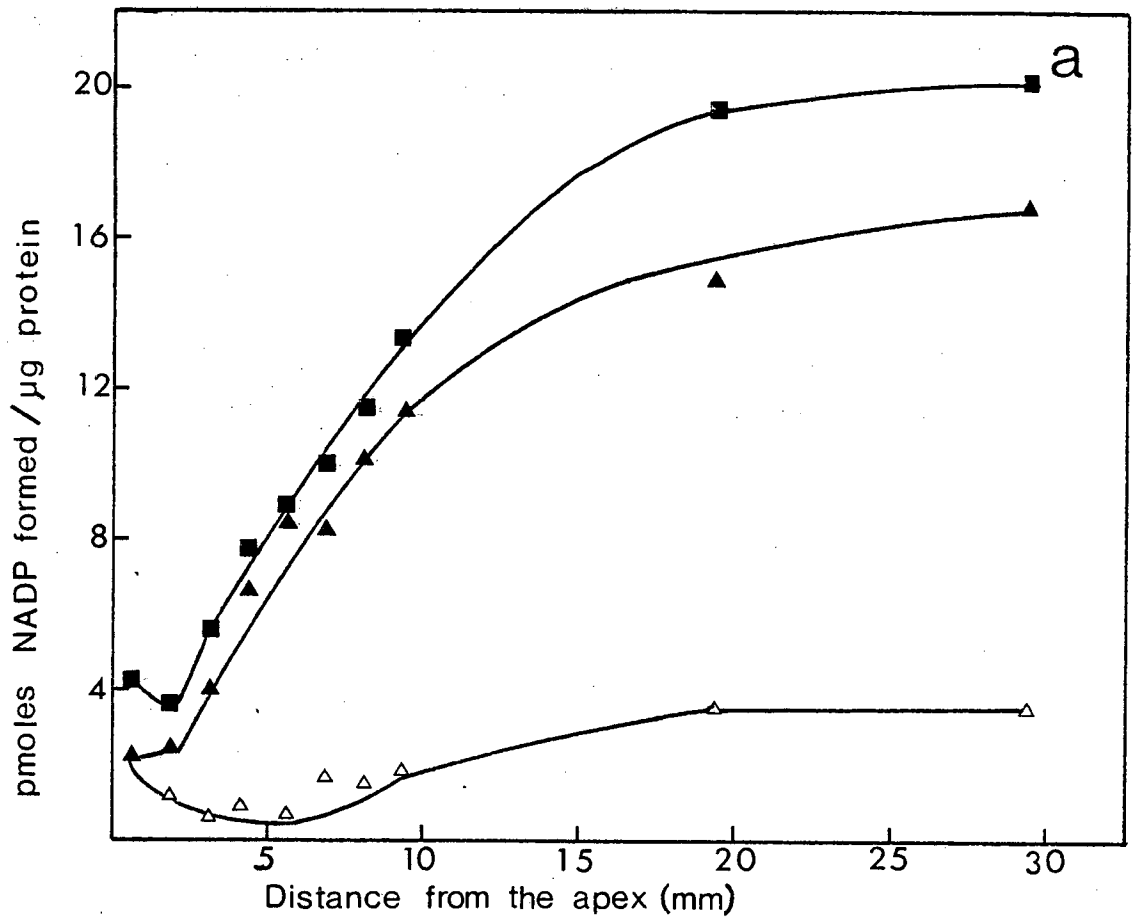
Figure 4:2:10. Developmental changes in NAD kinase activity in the root apex

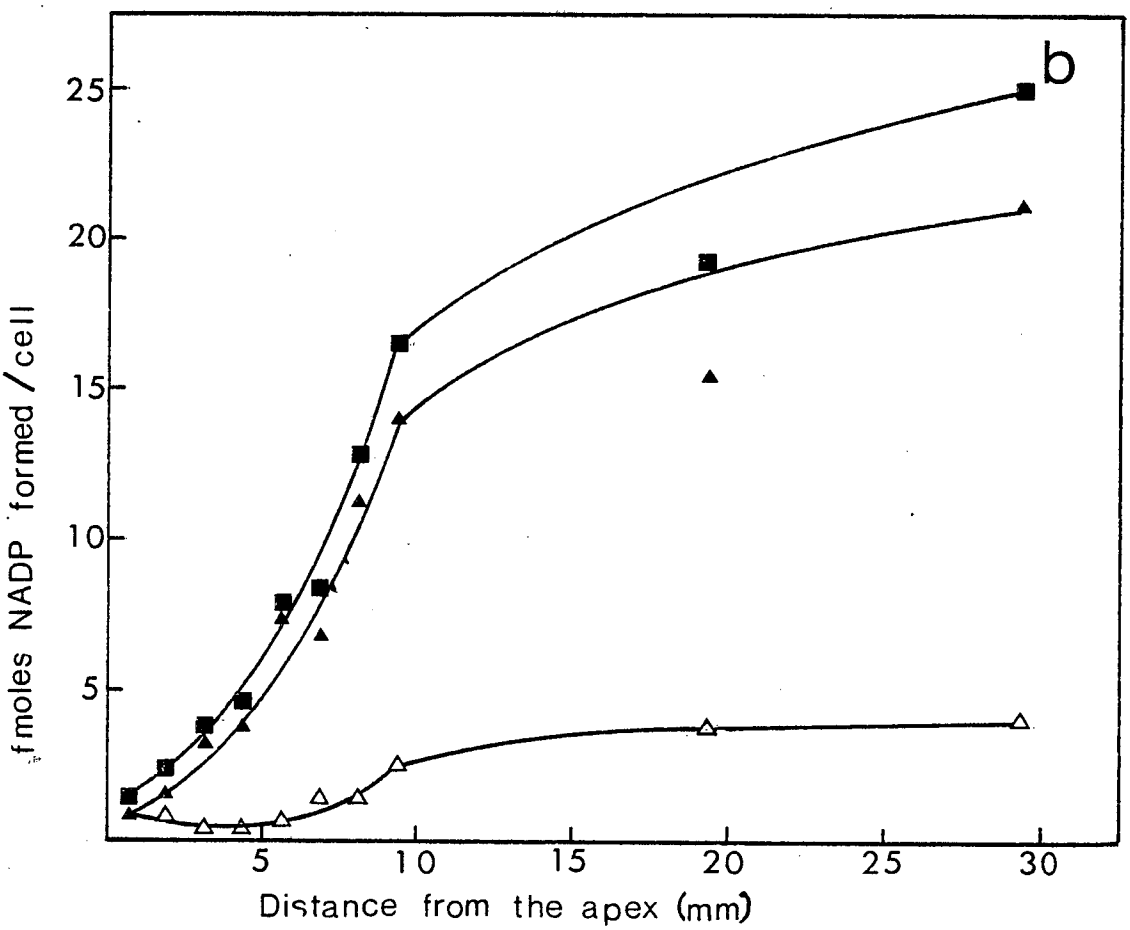
NAD kinase was extracted from serial 1.25mm sections of pea roots as described in chapter 2 section 2 H ii c, and assayed according to a modification of the method of Muto and Miyachi (1977) as described in chapter 2 section 2 I ii.
Total potential NAD kinase activity (——■——) was estimated in the presence of saturating amounts of bovine brain calmodulin and 1mM $CaCl_2$. Calmodulin-independent activity (——△——) was estimated in the presence of 2mM EGTA or 100μM trifluoperazine. Calmodulin-dependent activity (——▲——) was estimated by subtraction of the calmodulin-independent activity from total potential activity. NAD kinase activity is expressed as pmoles NADP formed/30 minute incubation.

a) Developmental changes in NAD kinase activity per unit protein.

b) Developmental changes in NAD kinase activity per cell.

Fig.4:2:10.


a
20
16
12
8
4
pmoles NADP formed / µg protein
5
10
15
20
25
30
Distance from the apex (mm)


b
25
20
15
10
5
fmoles NADP formed / cell
5
10
15
20
25
30
Distance from the apex (mm)

the plastid envelope, synthesis of NADP has to take place in both compartments. As NADP-requiring enzymes are also found in both the cytoplasm and plastids, regulation of NADP levels in both compartments might be expected to occur. As the two forms of NAD kinase appeared to be present in different cell compartments (chapter 3, section 3 A), it was therefore of interest not only to investigate whether these two forms were present in roots, but also whether they showed a different pattern of activity throughout the root apex.

As described in chapter 3 section 3 A ii and iii, both forms of the enzyme were found both by ion exchange chromatography and by affinity chromatography to be present in roots. As can be seen in figure 4:2:10 they did in fact show a different pattern of activity and the calmodulin-independent form contributed a much greater proportion of potential activity than it did in shoot tissue. The calmodulin-dependent form when fully activated with calmodulin was at a minimum in the apical 2mm, increased rapidly thereafter undergoing a 20-fold increase/cell by 10mm, and continued to rise in activity throughout the entire apical 3cm. On the other hand, the calmodulin-independent form, while contributing 50% of the potential total activity in the apical millimetre, fell to 3mm from the apex per µg protein, then increased slightly to a maximum by 20mm, remaining constant thereafter. Calmodulin-independent activity/cell remained roughly constant until about 6mm from the tip after which it rose slightly to 20mm, providing 16% of total potential activity by 30mm from the apex.

The change in calmodulin-dependent NAD kinase activity did not correspond closely to the amount of calmodulin present in several respects. The main differences occurred in the apical 2mm where calmodulin/cell or /µg protein was ten times higher than in the subsequent millimetre, whereas the calmodulin-dependent NAD kinase activity was slightly lower in the apical millimetre than in the following millimetre. The other main region of difference occurred after 10mm from the tip, where calmodulin both per cell and as a proportion of soluble protein remained roughly constant, although calmodulin-dependent NAD kinase continued to rise. The variations in degree of increase/

cell of the two proteins also differed in that calmodulin-dependent NAD kinase activity increased 20-fold over 10mm whereas calmodulin increased only 13-fold/cell within the apical 10mm. The total concentration of calmodulin did not appear to be limiting to any significant extent in any of the sections, as virtually no increase in activity occurred with addition of calmodulin in any of the sections examined. This again contrasts with the shoot in which addition of calmodulin to the crude homogenate consistently increased NAD kinase activity to about 130% of its original activity. To investigate whether calmodulin limits NAD kinase *in vivo*, however, it would be necessary to compare the intracellular distribution and concentration of calmodulin with that of NAD kinase and ionised calcium.

Although the concentration of calmodulin-dependent NAD kinase and its activator calmodulin did not follow entirely similar patterns, total concentration of calmodulin did not appear to be limiting for *in vitro* NAD kinase activity. The greatest discrepancies in concentration of calmodulin and the potential activity of calmodulin-dependent NAD kinase therefore appeared to occur in the apical millimetre where calmodulin-dependent NAD kinase was at a minimum, whereas calmodulin was in relatively high concentration before falling to a minimum; and beyond about 8mm from the apex where calmodulin remained approximately constant in concentration, as a proportion of protein, and per cell; whereas calmodulin-dependent NAD kinase continued to rise. It is not known whether calmodulin might be limiting to calmodulin-dependent NAD kinase *in vivo*.

The ratio of calmodulin-independent NAD kinase activity to potential calmodulin-dependent NAD kinase activity not only varied throughout the root apex, decreasing from 50% of total potential activity in the apical millimetre to 16% by 30mm, but also was higher than that observed in shoot tissue, where calmodulin-independent NAD kinase activity constituted only 10% of total potential activity in light-grown tissue. It is possible that the altering ratio of the two forms, as well as alteration in total NAD kinase activity, has a developmental significance. The functional significance of alteration in concentration of calmodulin is not understood, but it does not

appear to be limiting of calmodulin-dependent NAD kinase activity.

d) Summary

The results obtained for concentration of individual proteins provided further evidence that differentiation is accompanied by relative changes in protein content, and that alteration in concentration and activity may have a functional significance. However, the results showed further inconsistencies with the model of root differentiation proposed by Brown (1963).

1. A regulatory, non-enzyme protein, calmodulin, was found to alter in concentration and in amount/cell considerably throughout differentiation, and therefore potentially the concentration of a regulatory protein might affect proteins involved in differentiation.

2. Calmodulin-binding proteins were found in pea roots, and calmodulin might therefore have a functional role in cell development in the root.

3. As mitosis, in which calmodulin is involved, and amount of calmodulin-dependent NAD kinase both altered at different stages of development, the function of the regulatory protein calmodulin probably varies throughout differentiation.

4. As calmodulin-dependent NAD kinase altered not only in concentration throughout the root tip but also as a proportion of total potential NAD kinase activity owing to a far greater increase in the calmodulin-dependent isozyme, an enzyme that can be regulated post-translationally may be involved in differentiation.

5. Alteration in enzyme activity might be affected by concentration or compartmentation of a regulator, and post-translational control may therefore operate.

6. In support of the observations on general protein changes by two-dimensional electrophoresis, calmodulin was found to alter at different distances from the apex in different tissues, therefore no evidence was obtained for the hypothesis that proteins

involved in differentiation altered at the same distance from the apex in all tissues varying only in the rate and degree to which they alter.

7. No evidence was obtained for the hypothesis that individual proteins increased in the 'cell expansion' zone and subsequently stabilised or decreased, as increases frequently occurred at different distances from the apex and as some proteins decreased during the 'zone of expansion'.

8. Little evidence for transcriptional control of gene expression was obtained, and it appears that control is largely at the post-transcriptional level.

(iv) Concluding comments

Differentiation was found to be accompanied by extensive changes in protein concentration, protein composition and protein synthesis. Apparent protein synthesis/cell decreased considerably as cells aged although protein content/cell rose slowly, indicating that an increasing proportion of protein in the cell had a low turnover rate. This occurred earlier in the stele than in the cortex, possibly as a result of an increase in structural protein, and cessation of nuclear-directed protein synthesis.

The apparent reduction in protein synthesis was found not to be a result of either a reduction in rRNA or in *in vitro* translatable mRNA, as both increased 3-fold/cell while protein synthesis dropped to negligible levels. Furthermore, the alteration in composition of *in vitro* translation products was minimal as cells aged, although considerable variation in protein composition and protein synthesis occurred both as cells aged and between cells of different tissues. These observations indicated that post-transcriptional control of protein synthesis operated in differentiation in the pea root apex.

Three proteins analysed in further detail, the regulatory protein calmodulin, and two isozymes of NAD kinase, one of which is regulated post-translationally by calmodulin and calcium and the other apparently independent of calmodulin, confirmed that proteins not only varied during differentiation in amount, but

also in relative concentration. At least one of them, calmodulin, also varied between tissues both in concentration and in the distance from the apex at which change in concentration occurred. The variations in these proteins coupled with knowledge of their functions in root tissue, indicated that variations in these proteins might have some developmental significance in the root apex.

Several inconsistencies with the model of root differentiation proposed by Brown (1963) were found. Major inconsistencies were the manner in which proteins were proposed to vary during differentiation; the role of regulatory proteins in differentiation; and post-transcriptional regulation in general and post-translational regulation in particular were found not only to be very probably involved in differentiation, but probably to be the major forms of control of protein composition and activity in the root apex. As described in chapter 4 section 1 E, the results also contrast with the classical theory of root development on which Brown's molecular model of differentiation was based. The root does not appear to consist of successive physiological zones involving qualitatively and inherently different processes. Cell division, expansion and differentiation occurred at all levels of the apical 30mm examined.

The implications of these observations will be discussed in chapter 5.

D I S C U S S I O N

CHAPTER 5

1. INTRODUCTION

The primary aims of this study were to link molecular and structural events during early stages of cell differentiation at the root apex, and to investigate gene expression during early differentiation in relation to the epigenetic/transcription theory of differentiation in the form proposed by Brown (1963) for the root apex. The results from both anatomical and biochemical studies raise many interesting points on the nature of cellular differentiation and its control in the root apex.

In this chapter, initially, the validity of the main techniques will be evaluated. The results of certain aspects of the study that have relevance to the development of the root will then be summarised, and their implications for differentiation and development at the root apex discussed. Finally, this study has indicated that there are several areas of research that it may be worthwhile to pursue. Suggestions for preliminary experiments to investigate three main areas are therefore provided at the end of the chapter.

2. TECHNIQUES

The limitations of the techniques used and the attempts to overcome some of these have been discussed in relevant sections of chapters 1,3 and 4. The main disadvantages of the techniques are summarised below.

A. Tissue was obtained by a combination of the serial sectioning technique and microdissection of groups of tissues from these sections. Some loss of soluble protein will have occurred by this method, and some degree of cross-contamination of different segments with soluble protein will also have occurred. This will be proportional to the volume, particularly the length, of cells at the exposed surface, and might therefore be expected to become worse in more basal segments. Furthermore, although separation of groups of tissues could be readily achieved in more basal segments, in apical sections the tissues did not separate so discretely, and each group of tissues in these sections will

therefore contain a small proportion of contaminating cells from other tissues.

The separation of tissues achieved was very limited owing to the difficulty in separation of pericycle and endodermis from the cortex and stele, and to the radial distribution of vascular tissues within the stele which were not readily detectable until 20-30mm from the apex.

However, despite the fact that variations in many individual tissues will be obscured, all characteristics examined showed tissue or tissue-group - dependent distributions at the same transverse level indicating that partial dissectioning of serial sections may provide useful information.

Estimations of cell division, shape, size and number in successive serial segments were obtained by anatomical studies, as this provided more reliable estimations than other methods, and as it provided information on individual tissues, and on cell progression from the apex of the root proper.

B. The validity of the analysis of protein composition and protein synthesis by the two-dimensional polyacrylamide gel electrophoresis technique has been discussed in chapter 3. Although many precautions were taken to minimise known artifacts including the removal of nucleic acids and prevention of aggregation of proteins, both of which appeared to interfere with electrophoresis of pea root proteins, it is possible that artifacts may have occurred.

In a comparison of protein composition between different segments it is important to assess whether apparent differences between tissues might have arisen as a result of different conditions that interfered with extraction and electrophoresis. By comparison of protein samples that had been homogenised separately and together, it was possible to demonstrate that if conditions producing artifacts were present in the pea root, they did not appear to differ between different root segments.

Extractability of protein may, of course, have varied between sections. Homogenisation and electrophoresis of root segments by a variety of methods supported the observation that numerous quantitative and qualitative changes occurred both

between different groups of tissues and during tissue development. However, proteins could not be matched on gels using different methods of extraction, and comparison of extraction of individual proteins by a variety of methods would have to be compared to assess potential differences in solubility of individual proteins between sections.

Although comparison of total unidentified soluble protein between different tissues does not provide information on the precise developmental significance of change in protein composition; nevertheless, useful information may be obtained on the degree of change, and on the nature (whether qualitative or quantitative) of change.

C. However, in order to obtain a more reliable estimation of change in protein composition and to investigate the developmental significance of changes in protein levels, the distribution and concentration of calmodulin was analysed both by polyacrylamide gel electrophoresis and by biological activity, with investigation of extraction procedures and conditions potentially interfering with the assays.

In estimating calmodulin by two-dimensional PAGE, purified plant calmodulin could not be identified on polyacrylamide gels, probably due to limited proteolysis during extraction or to modification of electrophoretic mobility. Calmodulin was therefore tentatively identified indirectly on polyacrylamide gels of crude homogenates by comparison with the electrophoretic mobility of bovine brain calmodulin, and by the unique characteristics of the calcium-dependent alteration in electrophoretic mobility. The validity of this approach is discussed in chapters 3 and 4. Differences in extractability or in modification of calmodulin between different zones of the root were estimated by homogenisation of zones separately and together, and by co-homogenisation of the crude homogenate with purified bovine brain calmodulin. No differences in mobility or quantity of either the putative calmodulin or bovine brain calmodulin could be detected. Attempts to develop a technique to identify calmodulin more readily by binding to (^{3}H) trifluoperazine were only partially successful and were therefore not utilised.

In the estimation of calmodulin by NAD kinase assay, both proteins were purified to remove interfering factors including calmodulin-binding proteins, as discussed in chapter 3. A calmodulin-like protein was purified almost to homogeneity by the criterion of two-dimensional electrophoresis. Although it was demonstrated to possess many of the characteristics of calmodulin, including the ability to activate NAD kinase which has no other known activator, it has not been conclusively demonstrated to be calmodulin. Functionally, however, it appears to be identical to calmodulin; and this is perhaps of greater relevance in this study than of the demonstration of calmodulin itself.

As described in chapter 3, extraction of calmodulin-like activity varied considerably depending on the conditions employed. This was suggested to be due largely to binding to membrane-bound calmodulin-binding proteins in the presence of calcium, as addition of EGTA and salt to the homogenisation medium dramatically increased extraction. It is possible, however, that as some proteins may bind to calmodulin independently of calcium, and in at least some cases may not be released either with EGTA or high salt concentrations (Hooper and Kelly, 1984), it is possible that not all calmodulin was extracted from the tissue. Differences in extractability due to non-specific binding and differences in modification of activating ability between different segments were examined by homogenising sections separately and together, and by co-homogenisation of pea tissue with bovine brain calmodulin. Differences between these treatments were found not to be significant.

As purified pea calmodulin was not available, bovine brain calmodulin was used to calibrate the NAD kinase activator. The activating ability of pea and bovine calmodulins may not have been identical however, possibly tending to overestimate the concentration of pea calmodulin present. This, in conjunction with the possibility of modification of pea calmodulin during extraction which would underestimate the apparent concentration of calmodulin, indicated that quantitation of pea calmodulin may not reflect the actual amount of calmodulin in the sample. However, as these artifacts would probably tend to affect

calmodulin from different root sections equally, the relative values of calmodulin in different root sections were compared.

I was therefore not able to demonstrate the presence of calmodulin itself in pea root tissue. However, a protein with unique calmodulin-like proporties was observed on polyacrylamide gels of crude homogenates and was compared with a protein that was functionally identical to calmodulin and which possessed many other characteristics of calmodulin. Despite the differences in methods of extraction and assay of calmodulin the results were remarkably consistent between the techniquesproviding some reassurance that the values obtained were reasonable estimates of the relative, if not quantitative, distribution of calmodulin in the root apex. At least one protein with unique calmodulin-like activity was therefore demonstrated to be present in pea root tissue, and to vary during root development.

D. NAD kinase distribution in the root apex was assayed by biological activity. The protein was too unstable to permit purification to homogeneity prior to assay, and could therefore not be purified for PAGE or enzyme assay. NAD kinase was, however, partially purified to eliminate inhibitory influences on activity. Assays were carried out on all samples in a series at the same time after extraction to minimise variation in extraction caused by differential degrees of loss of activity.

Extractability of NAD kinase, as with calmodulin, was greatly affected by extraction conditions. Again, like calmodulin, EGTA and high salt concentrations were required for solubilisation of a large proportion of the activity, probably due to release from membrane-bound or organellar sites and from membrane-bound calmodulin. Although high salt concentrations and EGTA were included in the homogenisation medium, differential extraction of NAD kinase may affect the assay of the enzyme. The possibility of modification of NAD kinase during extraction has been discussed in chapter 3 section 3.

E. *In vitro* translation was used as a preliminary method of observing changes in mRNA composition during differentiation. Many artifacts may occur with this method, and the complexity and abundance of *in vitro* translation products need not reflect the complexity and relative abundance of input mRNA species.

Several precautions were taken to minimise artifacts as discussed in chapter 3, while *in vitro* translation products under a variety of translation conditions were examined. It was found that translation of several products could be affected by different ionic conditions, and a small effect on translation of specific mRNA species could be detected with increasing amounts of mRNA.

It should be noted, however, that artifacts such as incomplete translation, termination readthrough, misreading of the message, translation of incompletely processed mRNA species, and differential efficiency of translation of mRNA *in vitro* and *in vivo* due to different translation conditions, would generally be expected to lead to an increase in heterogeneity of translation products. In view of the observation that *in vitro* translation products were remarkably uniform between different sections, it seems probable that translation was not being affected differentially in different tissues.

No attempt was made to match *in vivo* and *in vitro* translation products, due to the likelihood of incomplete processing of *in vitro* translation products. Apparent specific changes in mRNA species may therefore not correspond to specific changes in *in vivo* translation or final protein concentration. Calmodulin, however, was tentatively identified in translation products by its unique characteristics of a calcium-dependent mobility shift; and by calcium-dependent binding of radioactivity to a phenothiazine affinity column. Although the *in vitro* translation system may have produced pI' and MWt values for translation products differing from the *in vivo* product; the *degree* of developmental changes in *in vitro* translation products could be compared to the degree of developmental changes occurring in protein composition and protein synthesis.

It is possible that the relative homogeneity of translation products in different sections was due to an anomaly of the wheatgerm system in that it selected certain mRNA species which it could translate with a certain efficiency. However, if this were so, it could be argued that differential translatability would also be likely to occur *in vivo*, and would further indicate that post-transcriptional control was operating *in vivo*.

Finally, several points should be noted. Firstly, only a small proportion of total mRNA species will have been observed. It is possible that differences occurred between developmental stages in mRNA species not translated sufficiently *in vitro* to be detected; while mRNAs translated in plastids or mitochondria will not have been observed at all. Secondly, due to the rapidity required in freezing tissue for RNA extraction to prevent nuclease activity, it was not possible to dissect individual tissues from successive root segments. It is therefore possible that differences in *in vitro* translation products may be detectable between tissues if not between different stages of differentiation. Thirdly, at best, only accumulation of mRNA may be detected. *In vitro* translation may not distinguish between transcriptional control, change in translatability of mRNA, and control of mRNA degradation.

3. DEVELOPMENTAL CHANGES IN THE PEA ROOT APEX AND THEIR IMPLICATIONS FOR THE MODEL OF ROOT DEVELOPMENT PROPOSED BY BROWN (1963, 1964)

A. Introduction

As estimated by two-dimensional electrophoretic analysis of total and individual proteins and biological assay of individual proteins, early differentiation in the pea root apex was found to be accompanied by extensive changes in protein composition. Differences in protein composition were detected both between different tissues and during tissue development. This is consistent with the hypothesis that changes in protein composition accompany differentiation. However, the changing pattern of protein composition did not appear to reflect that proposed by Brown (1963, 1964). Neither did the manner of control of differentiation by protein nor did the control of protein composition appear to conform to the regulatory mechanisms specified by Brown. A study of the cytological characteristics of growth, division and differentiation in individual tissues also showed numerous inconsistencies with the manner of cell differentiation and its control as specified by the model. As the structural and biochemical aspects of differentiation were integrated into a coherent and interdependent whole in this model, to a large extent unjustified assumptions and misconceptions of one aspect of differentiation led to further misconceptions in other aspects. Developmental changes observed in a number of anatomical and biochemical characteristics and their relevance to the model of root differentiation of Brown will be further discussed in the following sections.

In the model of root differentiation of Brown, protein and enzyme composition were proposed to vary in a specific and strictly regulated manner, and to be ultimately responsible for cell differentiation. The model specified several aspects of the pattern of the changes in enzyme activity, and these will be discussed below.

B. Developmental changes in protein composition and activity and their significance for differentiation in the root apex

(i) General changes in protein composition during differentiation

According to the model, proteins increase beyond the meristem in the 'zone of elongation', reach a maximum, and then decline or remain at a constant level. Similar patterns of protein development are proposed to occur at the same distance from the apex in all cell types. Different tissues are proposed to arise within this general framework of change by variations in the extent to which and in the rate at which proteins alter.

Brown developed this concept on the basis that cells matured at the same rate; that structural variations between cell types were negligible during expansion and early differentiation; that zonal patterns of development of *in vitro* activities of several enzymes were consistent, and that such uniformity of trends observed in a number of biochemical characteristics would not be expected if the course of development in different cells were highly variable. As discussed in chapter 1 section 1 B and chapter 4 section 2 the reasoning behind the latter assumption is faulty, and experimental support extremely poor. Zonal analysis and analysis of segments cultured *in vitro* are clearly inadequate both on theoretical and experimental grounds for the investigation of differentiation in different tissues. I therefore examined protein composition in a number of tissues as differentiation progressed.

From two-dimensional polyacrylamide gel electrophoresis of proteins from different tissues during development it appeared that although many proteins did undergo a similar course of development in different tissues, many did not. Neither did all proteins increase during cell expansion, and decline or stabilise thereafter. Several proteins continued to increase in certain tissues, or were not observed until a late stage of cell maturation. Other proteins decreased during expansion and early differentiation. In several cases, proteins remained constant in certain tissues while increasing or decreasing in others, or increased in certain tissues while decreasing in

adjacent tissues. Examination of individual proteins by electrophoresis and biological activity also showed inconsistencies with the model: the concentration of the activator protein calmodulin not only decreased from the apex, but altered differentially at different distances from the apex in different tissues, falling initially in the epidermis and cortex and later in the stele. A subsequent increase in concentration appeared to occur exclusively in the cortex. Potential calmodulin-dependent NAD kinase activity on the other hand was observed to increase throughout at least the apical 30mm. Protein composition therefore did not follow uniform trends in different tissues, nor did all proteins increase in the 'zone of expansion' and decrease or stabilise thereafter.

Many developmentally changing processes such as cell wall synthesis, vacuole ontogeny, vacuole expansion, and plastid and mitochondrial development progress in a linear manner from the root apex and are observed in different cell types. Progressive changes and a similar pattern of protein development in different cell types might therefore indeed be expected to occur but to different extents and at different rates as specified by the model. However, although a number of proteins did in fact follow the prescribed pattern, the many discrepancies between the model and the observed development of protein patterns may be attributed to three main considerations. Firstly, contrary to Brown's view, cells do in fact mature at greatly differing rates as may be seen from chapter 4 section 1. Xylem, for example, matures slowly. Secondary wall deposition occurs beyond about 10mm from the apex, while lignification occurs at around 20mm from the apex. Sieve elements on the other hand mature rapidly, the first element developing thickened walls by 300μ and maturing within 4-500μ of the quiescent centre/root cap junction. By 3-4mm from the apex, a considerable proportion of protophloem and metaphloem sieve elements appeared from EM studies to have matured. Secondly, differentiation includes development of specialised structures in particular cell types, while selective or complete loss of cytoplasmic and/or nuclear material also occurs in certain specialised cells. Thirdly, although protein content was found to increase per cell

during cell expansion, the rise was found not to be so great as indicated by the data of Brown, while there is no *a priori* reason to suppose that all proteins increase albeit differentially, during cell expansion and subsequently peak or decline at the end of expansion. Development of protein patterns would therefore not be expected to be either qualitatively, spatially, or temporally so uniform as proposed in the model.

(ii) Quantitative and qualitative changes in protein composition during differentiation

Brown proposed that only quantitative changes in proteins occurred during differentiation. However, several tissue-specific and stage-specific proteins were detected by two-dimensional polyacrylamide gel electrophoresis. Although it is possible that several of these proteins were in too low a concentration to be detected in all tissues rather than being completely absent from some tissues, it seems probable that at least a few of these proteins are unique to specific aspects of differentiation, being involved either in the specification or the expression phase of differentiation. Additionally, at least several proteins will be preferentially lost during differentiation of certain stelar cells.

However, qualitative changes might be expected at least, for example, in development of sieve elements. During maturation of these cells, the nucleus breaks down and selective loss of cytoplasmic material presumably including nuclear-encoded protein occurs. Specialised structures such as the secondary cell wall and sieve tube reticulum also develop. Xylem cells develop thickened and lignified cell walls, and subsequently undergo total loss of cytoplasmic components. Several substrate-specific isozymes are known to occur in plants. These include the lignin-specific isozyme of 4-coumarate:Co A ligase (Hahlbrock and Grisebach, 1979) which might be expected to be absent in early stages of differentiation but present during lignin formation in secondary walls, particularly in xylem and phloem sclerenchyma cells.

Although it should be noted that selective or total loss of

nuclear and/or cytoplasmic material in specialised stelar cells will have been largely obscured by the presence of stelar parenchyma cells in the tissue samples, and will thus artifactually conceal qualitative changes in protein composition, most of the changes in protein composition did, however, appear to be quantitative rather than qualitative. This presumably largely reflects the fact that many of the differences at the structural and ultrastructural level involved differences in the proportions of cellular components including the cell wall, nucleus, cytoplasm, vacuole, plastids, mitochondria, ribosomes and Golgi bodies; or differences in the size or in the degree of development of internal structure of plastids and mitochondria rather than qualitative differences in structure. It is also known that many of the compounds accumulating in, or characteristic of, a specific tissue, are present in more than one cell type. Others are synthesised utilising metabolic pathways common to several biosynthetic activities. Lignin, for example, is particularly high in cell walls of mature xylem, phloem fibres and endodermis; however, it is found in walls of all other cell types to a lesser extent. Furthermore, lignin is synthesised from phenylpropanoid units derived from the shikimic acid pathway. Specific isozymes direct these units to lignin synthesis at a very late stage in its biosynthesis (Hahlbrock and Grisebach, 1979). Thus, although enzymes involved in the shikimic acid pathway increase during lignin formation, it is a quantitative change, and almost all the enzymes are involved in production of other compounds including cinnamate esters and flavin compounds.

Many compounds present in abundance in specific tissues such as callose (β-1,3-glucan) a component of mature sieve tubes; β-1,4-glucan, a component of root cap slime; and other β-1,3- or β-1,4-glucans including cellulose (Moore and Stone, 1972), are all synthesised initially from the same compound, in this case UDP-D-glucose. Thus, again, almost all enzymes involved in the synthesis of these tissue-associated compounds would not be expected to be tissue- or stage-specific. Similarly, a major enzymic reaction involved in the synthesis of both suberin, abundant in endodermal cell walls, and cutin, associated with epidermal cell walls, is the ω-hydroxylation of fatty acids

(Soliday and Kolattukudy, 1977). Peroxidase appears to be localised almost entirely in the root cap, hypodermis, endodermis and phloem in *Allium* root tips (Goff, 1975), and again this enzyme is not confined to a single tissue although present in widely differing concentrations.

By one-dimensional polyacrylamide gel electrophoresis, a few unusual protein bands from sieve tube exudate were found to dominate the protein composition of this material (Beyenbach *et al.*, 1974; Kleinig, 1975; Weber and Kleinig, 1971). However, these proteins were not compared to those either from other phloem or non-phloem cells and it is therefore not clear whether they are tissue-specific, or are rather quantitatively relatively high in sieve tubes.

Other proteins with different functions during differentiation, such as tubulin, microfilaments, E.R., and intramembrane proteins, may alter in degree of polymerisation, form of assembly, and/or intracellular distribution, rather than alter in concentration during differentiation.

Thus, quantitative differences in protein concentration appear to be predominant during differentiation, probably reflecting the relative predominance of quantitative structural and biochemical differences in the root apex. However, qualitative changes do occur in contrast to the specifications of the model of differentiation of Brown.

(iii) Is differentiation regulated by enzyme concentration?

One of the major aspects of the model of root differentiation of Brown is the directing of growth, and hence differentiation, by enzyme proteins. Enzyme concentration was further proposed to be the crucial factor in controlling enzyme activity, with differential control by activators and inhibitors having no part in differentiation. Enzyme concentration was proposed to be regulated at the level of transcription.

Although these are central aspects of the model, no evidence was presented for the proposals, which were developed on the basis of observations on changing *in vitro* enzyme activities in the region of maximum rate of root elongation and on alterations in the gross composition of RNA during differentiation.

The presence of activators or inhibitors of enzymes under investigation as control experiments did not even appear to have been carried out. It is clear, however, both from my own studies and those of others that the assumptions involved in developing these proposals were not justified.

In vitro enzyme activity need not reflect *in vivo* activity. Nor need *in vivo* or *in vitro* activity reflect the concentration of the enzyme. Examination of protein concentration and synthesis in pea roots by two-dimensional electrophoresis indicated indirectly that differential rate of degradation may regulate the levels of some proteins post-translationally. Post-transcriptional control of protein synthesis was also found to be widespread in the root apex and will be discussed in sections B iv-v and C. Enzyme activities might also be expected to be dependent not only on enzyme concentration, but also on such factors as pH, compartmentation of enzyme, cofactors, and substrate, compartmentation of regulated proteins and their regulators, and on ion levels including calcium and magnesium. Other proteins than enzymes might also be expected to be involved in differentiation, and proteins other than those involved in growth might also be involved in differentiation.

As transcriptional control of enzyme activity and the control of differentiation by enzyme protein were such important aspects of the model of control of differentiation, I investigated two proteins, both of which may be regulated post-translationally by non-enzyme regulators, and both of which in turn act as regulators of other proteins. One of these proteins, calmodulin, directly regulates a variety of enzyme, structural and contractile proteins, including proteins involved in growth and division. The other protein, NAD kinase, regulates indirectly a variety of metabolic pathways and individual enzymes by regulating locally the ratio of concentrations of NAD and NADP. Despite their activities being regulated, and despite regulating further activities, through mechanisms other than those specified by the model, both of these proteins might be expected to be involved in a number of ways in the processes of division, growth, and differentiation. The possible involvement of these proteins in differentiation was therefore investigated, and their potential function in the root apex is discussed in the following sections.

(iv) Developmental changes in the concentration of calmodulin, and the function of calmodulin during cell differentiation in the root apex

a) Developmental changes in concentration of calmodulin

As described in chapter 4 section 2, the concentration of calmodulin appeared to be both stage- and tissue-dependent. It was found in highest concentration in the root cap, where it was estimated at 170μM. The areas of highest concentration in the root proper appeared to be very closely correlated with meristematic areas in different tissues, while the concentration fell very rapidly immediately beyond the meristematic areas to a minimum of about 10μM. This is inconsistent with Brown's model in that calmodulin declined rapidly as rapid cell expansion commenced, and as the concentration varied between tissues at the same transverse level.

Two-dimensional polyacrylamide gel electrophoresis of (^{35}S) methionine labelled proteins indicated that the change in calmodulin concentration was probably paralleled by a change in its rate of synthesis. In view of the apparently minimal degree of transcriptional control of protein synthesis in the root apex, it seems possible that these specific changes in concentration and synthesis may be regulated at the post-transcriptional level. However, this cannot be assumed, as Means and Chafouleas (1983) have observed that changes in calmodulin mRNA as quantitated by hybridisation to cloned human cDNA, precede changes in calmodulin levels during the cell cycle in CHO/K1 cells.

An alteration in the concentration of a regulatory protein is therefore associated with differentiation in the root apex. According to Brown's model, such alterations should have no functional or limiting value in the control of growth, and thus have no part in the process of differentiation. However, the changes in concentration of calmodulin may indeed be relevant to differentiation as discussed below.

There is evidence to indicate, however, that the localised concentrations and distribution of calmodulin are generally more important than the total cellular concentration. Calmodulin is not normally in limiting concentration in cells. However, levels

of calcium in the resting cell are estimated to be about 10^{-7}M (Fiskum and Lehninger, 1982). This concentration is too low to activate calmodulin, and transient calcium fluxes, which may be induced by a variety of stimuli, including hormonal, tropic, and electrical stimuli, are required for its activation. The compartmentation of calmodulin in relation to calcium fluxes is therefore generally more important than the total cellular concentration. However, even if the changes in total concentration of calmodulin had no significance in differentiation, localised calcium fluxes will regulate activity of calmodulin post-translationally, and the non-enzyme regulator calcium would therefore be involved in regulation of activity of calmodulin-dependent proteins locally. Non-enzyme post-translational regulation of enzyme activity would therefore still operate. The potential function of calmodulin and the significance of total and localised concentration of calmodulin are discussed below.

b) The function of calmodulin during differentiation in the root apex

I. The range of proteins regulated by calmodulin in the root apex

As indicated previously, calmodulin regulates the activity of a number of structural and contractile proteins in addition to a variety of key enzymes. From calmodulin-affinity chromatography it appeared that at least 16 polypeptides from pea root tissue bound to the column, indicating that calmodulin may regulate a number of proteins in the root apex. As these polypeptides mostly differed from polypeptides from shoot tissue binding to the column, it is probable that calmodulin has some specific and different functions in these organs.

Four major and twelve-thirteen minor polypeptides were detected in the EGTA eluate from the column by two-dimensional PAGE. This may be an overestimation of the number of calmodulin-binding proteins in the root as some of the polypeptides may be different subunits of a single protein, while the possibility of non-specific binding to the column could be investigated by

calcium-dependent binding of fluorescent or radioactively labelled calmodulin to proteins from the column either in non-denaturing gels or in Western transfers of denaturing gels. One major polypeptide binding to the calmodulin-affinity column in shoot tissue may possibly bind non-specifically as it was observed to be (the only polypeptide) present in the calcium eluate immediately prior to EGTA elution. As it was extremely basic, this may represent non-specific binding to the highly acidic protein calmodulin. However, the possibility that it represented calcium-independent binding to calmodulin as observed with a very few proteins (Hooper and Kelly, 1984; Glenney *et al.*, 1980; Welsh *et al.*, 1982; Cohen *et al.*, 1980) cannot, at this stage, be ruled out. No such binding was observed in root samples, however.

Electrophoresis of proteins binding to a calmodulin affinity column may not, on the other hand, detect all calmodulin-binding proteins of the root apex. For example, calmodulin-dependent NAD kinase activity was found to bind to the column; however, no polypeptide of the MWt of NAD kinase was detected on the gels. Lack of detection by gel electrophoresis may be due to instability in the case of enzymes like NAD kinase; to weak binding by proteins such as tubulin (Sobue *et al.*, 1981b; Jemiolo *et al.*, 1980); to interference with binding to the column by endogenous calmodulin; to extreme pI or MWt values; or to a very low concentration of the protein in question.

Several of the calmodulin-dependent proteins likely to be present in the root apex include tubulin (Kumagai and Nishida, 1979), microtubule-associated proteins (Jemiolo *et al.*, 1980; Sobue *et al.*, 1981a), tubulin kinase (Burke and DeLorenzo, 1982), Ca^{2+} ATPase (Caldwell and Haug, 1980, 1981b), protein kinases (Hetherington and Trewavas, 1982; Salimath and Marmé, 1983), NAD kinase (Anderson *et al.*, 1980; chapter 4 section 2), lipoxygenase, superoxide dismutase (Leshem *et al.*, 1982), and the F-actin binding protein, caldesmon (Sobue *et al.*, 1981b).

It is clear that calmodulin may regulate a number of proteins central to cell function in the root apex.

II. The significance of calmodulin and non-enzyme proteins regulated by calmodulin to the model of root differentiation proposed by Brown (1963)

Two proteins, NAD kinase (chapter 4 section 2) and Ca^{2+} ATPase (Caldwell and Haug, 1980, 1981b; Oláh *et al.*, 1983) have been shown to be present in root tissue and to be dependent on calmodulin. A third protein, tubulin, is almost certainly dependent on calmodulin for polymerisation in roots as it has been found to be dependent on calmodulin in all organisms so far examined. These proteins will be discussed below.

As discussed in the following section, calmodulin-dependent NAD kinase varies considerably in activity during development in the root apex, and the ratio of the regulatable calmodulin-dependent isozyme to the non-regulatable calmodulin- and calcium-independent isozyme also alters due to the change in activity of the calmodulin-dependent form. The alteration in ratio of the isozymes indicates indirectly that post-translational regulation of NAD kinase activity may become increasingly important during differentiation, and may have a functional significance during differentiation, possibly both in relation to total NAD kinase activity and to compartmentation of activity. The total cellular concentration of calmodulin may in this case not be important to activity of NAD kinase as there is no correlation in the change in concentration of the two proteins. However, the localised concentration of calmodulin and the activation of calmodulin by transient compartmented fluxes of ionised calcium in relation to the compartmentation of calmodulin-dependent NAD kinase, almost certainly will regulate activity of this isozyme as discussed in section v. This is inconsistent with the proposition of Brown that activators are not involved in control of activity of enzymes involved in differentiation, and that enzyme concentration is the limiting factor in activity.

Regulation of tubulin polymerisation may be a major function of calmodulin in the root apex. Calmodulin is known to greatly increase the sensitivity of tubulin polymerisation to calcium to cause inhibition of polymerisation in the micromolar calcium range (Marcum *et al.*, 1978). This may occur by direct binding of calmodulin to the tubulin dimer (Kumagai and Nishida, 1979).

High molar ratios (2-8:1) of calmodulin to tubulin are required for regulation of assembly (Nishida *et al.*, 1979; Marcum *et al.*, 1978; Welsh *et al.*, 1979); much higher concentrations than those required for activation of any enzyme that calmodulin is known to regulate. The high concentration of calmodulin in meristematic areas of the root apex may therefore be associated with the requirements of regulation of tubulin assembly-disassembly, particularly in the control of cell division as described below. As microtubules are involved in a number of features of differentiation, the observation that the concentration of calmodulin may regulate microtubule activity is inconsistent with the proposition of Brown that the concentrations of regulatory proteins are not important in regulating activity of proteins involved in differentiation. It is also possible, however, that the high concentration of calmodulin in the meristem and root cap may reflect a large number or a high concentration of proteins regulated by calmodulin in these zones, rather than a specific requirement for a high concentration for particular regulatory activities.

Although variations either in the total concentration or in the intracellular distribution and localised concentration of calmodulin may be involved in differential regulation of activity of a number of proteins, it might be argued that these proteins are not involved in differentiation. However, at least some of these proteins have major functions in differentiation.

As discussed in further detail in section v, NAD kinase activity may regulate the activity, synthesis and/or intracellular distribution of a variety of compounds involved in cell development and differentiation that are dependent on NAD(H) or NADP(H), including lignin biosynthesis and biosynthesis of products derived from the pentose phosphate pathway. Regulation of the energy supply, supply of amino acids and fatty acids would also be expected to be partially involved in the regulation of rate of growth, a crucial vehicle for differentiation as described by the model of differentiation of Brown. Control of NAD kinase activity would therefore affect a number of processes involved in differentiation.

The degree of tubulin polymerisation and intracellular

distribution of microtubules are similarly involved in a wide range of activities associated with differentiation and morphogenesis. Microtubules are involved in cell wall formation through directing the movement of wall-forming vesicles (Doohan and Palevitz, 1980) which is dependent on calcium (Quader and Robinson, 1980) and possibly therefore on localised tubulin assembly/disassembly. Microtubules are also involved in orientation of cellulose microfibrils in the cell wall, including newly forming cell walls and secondary walls. As directioning of the orientation of microfibrils, and to a lesser extent wall-forming vesicles, by microtubules during interphase are believed to determine polarity of cell expansion in cells within tissues (Hardham, 1982) through determining future potential for directional growth (Hepler and Palevitz, 1974), the control of microtubule assembly and distribution are central not only to cell growth, but also to the polarity of cell growth and to root morphogenesis.

Calmodulin appears to be the mediator of the calcium-dependence of polymerisation of tubulin, either through direct binding to tubulin dimers (Kumagai and Nishida, 1979), phosphorylation of tubulin (Burke and DeLorenzo, 1982); regulation of microtubule-associated proteins (Jemiolo *et al*,, 1980; Sobue *et al*., 1981a) which affect the inhibitory activity of calmodulin on tubulin polymerisation (Jemiolo *et al*., 1980); or through involvement in microtubule organising centres, from which calmodulin has been isolated (Means and Dedman, 1980). Thus differential changes in cell shape and size may be at least partially under the direction of calmodulin-dependent polymerisation of microtubules.

Calmodulin may also regulate a number of aspects of cell division through regulation of microtubules or through regulating the levels of calcium locally. The mitotic spindle consists largely of microtubules. The ordered construction and disassembly of the spindle and the movement of chromosomes during mitosis are generally regarded as being largely dependent on the regulation of localised tubulin polymerisation under the control of localised calcium concentration (Kiehart, 1981) and microtubule organising centres. The mitotic cycle is therefore dependent on control of tubulin polymerisation.

It is well established from immunocytochemical data that calmodulin becomes highly concentrated in the mitotic apparatus during mitosis, and that it is a dynamic component of the mitotic spindle (Anderson *et al.*, 1978; Welsh *et al.*, 1978; Dedman *et al.*, 1982). Furthermore, it has been noted that calmodulin increases in concentration at late G2 and/or early S phase in dividing CHO/K1 cells (Chafouleas *et al.*, 1982). As localisation of calmodulin antibody alters from an initially diffuse cytoplasmic localisation at late prophase and early prometaphase to association with the highly calmodulin-sensitive cold-stable (Job *et al.*, 1981) kinetochore-to-pole microtubules during metaphase and anaphase when microtubule depolymerisation is taking place, retreating to the spindle poles as chromosomes separate (Welsh *et al.*, 1979), it has been suggested (Dedman *et al.*, 1982) that calmodulin depolymerises microtubules in the polar regions shortening the kinetochore-to-pole microtubules in the polar regions allowing chromosome movement to take place. Consistent with the proposition that calcium-activated calmodulin regulates microtubule depolymerisation locally is the observation (Wick and Hepler, 1980) that kinetochores appear to be calcium-free throughout mitosis thus maintaining the integrity of microtubules attached to chromosomes; whereas the membrane system at the spindle poles and within the half-spindles contain calcium which may be selectively sequestered and released.

Additional roles for calmodulin during mitosis and/or cytokinesis may include the regulation of spindle actomyosin (Herman and Pollard, 1979; Welsh *et al.*, 1979) through regulation of the F-actin binding protein caldesmon (Sobue *et al.*, 1981b), and myosin light chain kinase ATPase (Dabrowska *et al.*, 1978). Calcium sequestration and release from vesicles associated with the mitotic apparatus (Kiehart, 1981; Silver *et al.*,1980; Harris, 1975; Hepler, 1980; Wick and Hepler, 1980; Hawes *et al.*, 1981) also may be crucial for regulation of cell division. As Ca^{2+} ATPase activity associated with the mitotic apparatus varies during mitosis (Petzelt, 1972; Mazia *et al.*, 1972) and corresponds with variations in calcium uptake (Clothier and Timourian, 1972; Timourian *et al.*, 1974), it is possible that the calmodulin-dependent Ca^{2+} ATPase of root tips (Caldwell and Haug, 1980, 1981b) apparently associated with nuclei in meristematic root tip cells

(Sutcliffe and Sexton, 1969) may regulate localised concentration of calcium during cell division. This potentially will regulate chromosome condensation (Petzelt, 1972), mitosis (Kiehart, 1981), directing of cytoplasmic vesicles (Doohan and Palevitz, 1980) during cell plate formation, vesicle aggregation (Paul and Goff, 1973) during cell plate formation, the plane of cytokinesis (Timourian *et al.*, 1972, 1974) and polarity and asymmetry of cell division (Robinson and Jaffe, 1975).

The orientation, asymmetry and frequency of division are not only central to morphogenetic patterns and possibly to their control (Clowes, 1961; Lyndon, 1979; Bell and McCully, 1970), but are also dependent on position, tissue, and stage of differentiation within the root apex. Polarity of division is also involved in histogenesis, and indirectly in the maintenance of root shape and structure. Control of a variety of aspects of division by calcium and calmodulin through control of structural, contractile and enzyme proteins, and through localised electrophoretic effects, are therefore clearly features of differentiation.

Calmodulin may also be involved in control of cell expansion and geotropism through regulating calcium distribution via Ca^{2+} ATPase activation as discussed in section 4 C ii.

Many contractile activities in animal cells are regulated by calmodulin. It therefore seems possible that such activities may similarly be regulated by calmodulin in plant cells. As contractile proteins such as actin are known to be present in plant cells, activities such as vacuolar autophagy associated with specific stages of differentiation in the root apex might possibly be regulated by calmodulin.

It therefore appears that not only is calmodulin involved in differentiation, but that the functions of calmodulin vary during differentiation and development in the root apex. Changing levels of calmodulin may be related to specific functions in plant cell differentiation. However, the presence and concentration of calmodulin in a cell do not indicate whether it is in an active form. Neither does the presence of a calmodulin-binding protein indicate that it is activated by calmodulin *in vivo* in the tissue from which it was extracted. Activation of calmodulin-dependent processes is dependent on the localised

concentration and intracellular distribution of calmodulin in relation to the level of and distribution of calmodulin-binding proteins and ionised calcium. This will be further examined in section v.

As indicated previously, ion fluxes involving changes in concentration of calcium between 10^{-8}-10^{-6}M will be a major limiting factor in activation of calmodulin. However, the number of calcium ions bound per molecule of calmodulin is also believed at present to determine which calmodulin-binding proteins will bind preferentially to calmodulin, as these proteins have different affinities for calmodulin in its fully- and partially-calcium bound states. As all the calcium-binding sites of calmodulin have a k_d in the micromolar range, not only will major increases in calcium concentration affect the activity of calmodulin, but slight differences within the micromolar calcium range may further regulate the function of calmodulin, and may permit differential activation of several calmodulin-binding proteins within the same cellular compartment.

These considerations show further inconsistencies with the model of root differentiation of Brown, as not only may enzyme activity be regulated by activators rather than by enzyme concentration, and the concentrations of regulatory proteins may be involved in regulating differentiation, but structural protein (tubulin), contractile protein (actomyosin), regulatory protein (calmodulin) and a non-protein regulator (ionised calcium) may be involved in control of differentiation in addition to enzyme proteins. Furthermore, the total activity, intracellular distribution of activity, and concurrent function of such proteins, may be regulated at least partially by post-translational control mechanisms such as polymerisation, activation, compartmentation and intracellular movement, rather than by concentration.

(v) Developmental changes in NAD kinase activity, and the function of NAD kinase during root differentiation

a) Developmental changes in NAD kinase activity

It was observed that total potential NAD kinase activity increased three-fold per millimetre segment, seventeen-fold per

cell, and five to six-fold per unit protein between the apex and 20mm from the apex. This increase was due almost entirely to an increase in the potential calmodulin-dependent activity; and as a result, the ratio of potential calmodulin-dependent to calmodulin-independent activity rose approximately four-fold over this region. Again, this result is not entirely consistent with the specifications of the model of root differentiation of Brown. Firstly, NAD kinase continues to rise throughout at least the apical 30mm of pea root. Secondly, the developmental change in potential NAD kinase activity occurs in a regulatable enzyme. If the two forms of activity do indeed represent two different isozymes as discussed in chapter 3 section 3 A, the change in proportion of an apparently non-regulated isozyme to a rapidly regulatable form of NAD kinase may have a developmental significance. This is particularly interesting as it appeared from extraction characteristics observed in shoot tissue that the two forms may be present in different cell compartments, and NADP produced from the two isozymes may as a result be utilised for different functions.

According to the model of Brown, the increase in potential calmodulin-dependent NAD kinase activity should be reflected by an increase in *in vivo* activity, and the concentration of the enzyme should be the sole regulator of increase in activity. Activated calmodulin should therefore be present in saturating quantities throughout the root apex, or alternatively should not be involved at all in its activation. This will be discussed in the following sections. The implications of the presence and compartmentation of two forms of NAD kinase for differentiation will be discussed in sections b-d.

b) *In vivo* activity of NAD kinase in the root apex

The increase in potential calmodulin-dependent NAD kinase activity in the root apex refers to potential activity *in vitro* when fully activated by calmodulin and calcium. Whether such an increase occurs *in vivo* is not clear. There is indirect evidence to suggest that *in vivo* activity does increase, as the ratio of NADP(H): NAD(H) increases in more basal segments of the root due largely to a reduction in NAD and NADH both per segment and in concentration (Yamamoto, 1963). As the only

known pathway of NADP formation is the NAD kinase-catalysed phosphorylation of NAD by ATP, the relative proportions of NAD(H) to NADP(H) might be expected to be largely dependent on the activity of this enzyme. Although estimates of pyridine nucleotides are not available for the region of pea root that I investigated, two-day old *Vigna* roots were found to display a ten-fold increase in NADP(H):NAD(H) ratio and a thirteen-fold increase in NADP:NAD ratio between the apical 0.5mm and 5.5-6mm from the apex (Yamamoto, 1963). This indicates that the increase in potential *in vitro* activity of NAD kinase is accompanied by an increase in *in vivo* activity although it is not clear whether they occur to the same extent. This implies that the calmodulin-dependent form of NAD kinase is activated to at least some extent by calmodulin and that the increase in the calmodulin-dependent form has a functional value. This, however, does not necessarily imply full activation, nor does it imply that post-translational regulation does not influence NAD kinase activity even if the enzyme were in fact fully activated by calmodulin. The compartmentation of the two proteins, and localised fluxes of ionised calcium, will almost certainly be major regulatory factors in control of NAD kinase activity.

c) *In vivo* regulation of NAD kinase activity

It is well established that the NADP(H):NAD(H) ratio is rapidly altered on illumination of shoot tissue. The increase in NADP on illumination occurs in the chloroplast and not in the cytoplasm (Ogren and Krogman, 1965; Heber and Santarius, 1965; Oh-Hama *et al.*, 1963), and appears to be under phytochrome control (Dieter and Marmé, 1984). As NADP cannot cross the chloroplast envelope (Krause and Heber, 1976), presumably this increase in NADP is due to increased NAD kinase activity within the chloroplast. Phytochrome not only regulates NAD kinase activity (Tezuka and Yamamoto, 1972) but also may regulate calcium fluxes (Dreyer and Weisenseel, 1979; Hale and Roux, 1980; Dieter and Marmé, 1983). As illumination induces an influx of calcium to the chloroplast (Nobel, 1967; Muto *et al.*, 1982), while chloroplast calcium-calmodulin-dependent NAD kinase activity increases on illumination (Muto *et al.*, 1981, 1982) through decreasing the

k_m for NAD indicating there is an increase in the affinity of NAD kinase for NAD (Tezuka and Yamamoto, 1972), it seems reasonable, as has been suggested (Muto *et al.*, 1981; Muto, 1983; Jarrett *et al.*, 1982), that calmodulin-dependent NAD kinase activity increases on illumination as a result of a calcium influx to the chloroplast.

This provides evidence for post-translational regulation of the calmodulin-dependent NAD kinase in plant tissue *in vivo*, through regulation of ionised calcium fluxes. However, it should be noted that NAD kinase activity may be activated to similar extents both *in vivo* (Tezuka and Yamamoto, 1972) and *in vitro* (Tezuka and Yamamoto, 1972, 1975) by phytochrome. As activation of a cell-free extract is unlikely to act *via* alteration of ionised calcium, it is possible that regulation may be more direct, either through a direct effect on the enzyme to increase affinity for calmodulin, or through alteration of the distribution of NAD kinase or calmodulin, for example by release from thylakoids to the stroma. Phytochrome may act on the calmodulin-independent NAD kinase, but this is unlikely as light-induced activation is abolished by phenothiazines (Jarrett *et al.*, 1982). Although the mechanism of activation is therefore not clear, it does appear that post-translational regulation occurs, either through compartmentation of calcium, calmodulin, or NAD kinase, or through modification of existing NAD kinase.

NAD kinase located on the outer mitochondrial membrane (Dieter and Marmé, 1984) has also been demonstrated to be inactive in the absence of calcium and calmodulin, and to be activated in their presence while situated in the membrane. This also demonstrates the dependence of localisation of calcium and calmodulin in relation to NAD kinase.

Although control of ionised calcium levels in the root has not been investigated, normal cellular levels are generally accepted to be too low in the cytoplasm and probably in plastids for activation of calmodulin. Calmodulin-dependent activation of NAD kinase would therefore require elevated calcium levels. As the proportion of NADP:NAD does increase concurrently with the increase in the calmodulin-dependent isozyme, there is indirect evidence to indicate that free calcium is high enough

to activate calmodulin in roots, in at least some areas. However, it is not clear whether this reflects the normal level of free calcium in the relevant compartments with increase in NAD kinase activity due entirely to increase in amount of enzyme available in these compartments, or whether it represents a specifically-induced increase in localised calcium concentration. As growth substances, tropic and electrical stimuli are known to regulate calcium fluxes (Kubowicz *et al.*, 1982; Saunders and Hepler, 1982; Dieter and Marmé, 1981; Hale and Roux, 1980; Jaffe and Nuccitelli, 1976; Goswami and Audus, 1976), it is possible that such stimuli regulate intracellular calcium levels during root differentiation, and therefore potentially regulate the calmodulin-dependent isozyme of NAD kinase. It certainly seems unlikely that the increase in NAD kinase activity during differentiation is not under at least partial post-translational regulation, as an increase in calmodulin-independent NAD kinase, which might be expected if post-translational regulation were not functionally significant, did not occur.

d) Distribution of *in vivo* calmodulin-dependent and calmodulin-independent NAD kinase activities, and the relevance to differentiation

NAD kinase activity has been found in chloroplasts, the cytosol (Muto, 1982; Muto *et al.*, 1981; Jarrett *et al.*, 1982a), and the outer mitochondrial membrane (Dieter and Marmé, 1984). As indicated previously, I had observed that the calmodulin-dependent and calmodulin-independent activities required different extraction procedures. The calmodulin-independent activity could be extracted simply with Tris and PVPP, whereas the calmodulin-dependent form required harsher conditions for extraction. This indicated that the calmodulin-independent form may be present in the cytoplasm, and the calmodulin-dependent form either attached to membranes or present within organelles, probably including plastids. The greatest proportion of calmodulin-dependent NAD kinase activity in shoot tissue of most plants tested is present in chloroplasts (Muto, 1982) and activity may be increased by illumination. As a large proportion of pyridine nucleotides are present in plastids (Ogren and Krogman,

1965), and the level of NADP increases in the light, compartmentation of NAD kinase and its activation may be important in regulating activity of NAD kinase, at least in shoot tissue. Compartmentation may also regulate the function of the enzyme through control of the distribution of NAD and NADP. As NAD- and NADP-requiring enzymes are present in different intracellular distributions in plastids, cytoplasm and mitochondria (Quail, 1979; Schnarrenberger *et al.*, 1975; Leech and Murphy, 1976; Walker, 1976; Dennis and Miernyk, 1982; Yamada and Usami, 1976; Yamada *et al.*, 1974), the functions for which NAD and NADP are utilised will differ not only according to cell requirements, but also within different cell compartments. Differential regulation of coenzyme levels in different cell compartments may therefore qualitatively and quantitatively regulate the relative activities of different metabolic pathways in different compartments. Compartmentation of different isozymes of NAD kinase and different control mechanisms of their activation would therefore have a developmental significance. Localised calcium fluxes within a cellular compartment in addition to changes in total cellular calcium concentration would therefore also be involved in regulation of calmodulin-dependent NAD kinase, and in the functions for which the resulting localised alterations in coenzyme levels are utilised.

There is evidence that the alteration in ratio of pyridine nucleotides during differentiation in the root apex has a developmental significance. Several activities dependent on these coenzymes alter during root differentiation. For example, concurrently with the decrease in NAD levels in more mature sections of the root the NAD-dependent glycolytic pathway decreases in activity, while the NADP-requiring pentose phosphate pathway remains approximately constant as do NADP levels. As enzymes of both these pathways do not appear to alter in amount or ratio after about 6mm from the apex although an increasingly higher proportion of glucose is metabolised *via* the pentose phosphate pathway for at least the apical 40mm (Gibbs and Beevers, 1975; Fowler and ap Rees, 1970; Sutcliffe and Sexton, 1974), an alteration in coenzyme ratio may be the main regulatory factor involved in the continuation of this change beyond about

6mm from the apex. NAD and NADP are very low in concentration in the root apex (Yamamoto, 1963) and would be expected to be potentially rate-limiting in enzyme reactions. Alteration of the supply of NAD or NADP is also known to control relative activities of the pentose phosphate pathway and glycolysis in carrot root extracts and in yeast (Osmond and ap Rees, 1969; ap Rees and Beevers, 1960). These observations further support the proposition that coenzyme levels regulate these metabolic pathways in the root apex, particularly as the decreasing concentration of NAD in more basal segments will become increasingly rate-limiting.

A number of functions will be affected by alteration of coenzyme levels. For example, a decrease in the energy supply will result from a decrease in the NAD-requiring glycolytic pathway and the mitochondrial electron transport chain. The decrease in NAD and glycolysis in more basal segments may therefore reflect a decrease in energy requirements as cell division, expansion, and protein synthesis decline. A relative increase in the NADP-requiring pentose phosphate pathway which occurs in more basal segments will result in a relative increase in the production of NADPH for reductive biosyntheses and in production of carbon skeleton precursors for biosynthesis of a large variety of compounds such as ribose and deoxyribose for nucleic acid, erythrose as a precursor of the shikimic acid pathway and aromatic compounds,and pentose for coenzymes and polysaccharides. As NADPH (Hahlbrock and Grisebach, 1979; Pryke and ap Rees, 1976), NADP for activity of the shikimic acid pathway (Higuchi and Shimada, 1967a, 1967b), and the shikimic acid pathway itself, are all required for synthesis of lignin, it is possible that a major function of the relative increase in NADP and the pentose phosphate pathway as cells mature is the production of lignin during differentiation. This is supported by the observation that the pentose phosphate pathway is highly active in the stele and relatively inactive in the cortex, and that the increase in the stele coincides with the onset of lignification (Wong and ap Rees, 1971). Other major functions of the increase in NADPH with cell maturation may be the regulation of amino acid synthesis which is far higher in more

basal segments and which fluctuates according to requirements (Oaks, 1965); and fatty acid biosynthesis which is confined largely to plastids (Dennis and Miernyk, 1982; Yamada and Usami, 1975).

It is clear that NAD kinase indirectly regulates a number of functions associated with differentiation, including specific features of differentiation (lignin), energy supply and metabolites for cell growth (amino acids, fatty acids, carbon compounds), and reducing power (NADPH) controlling enzyme reactions involved in growth and differentiation. Compartmentation and localised regulation of NAD kinase activity in addition to control of total activity will therefore regulate the relative activities of a number of developmental features. These observations on NAD kinase are not consistent with the model of the control of gene expression during root differentiation of Brown. Calmodulin-dependent NAD kinase, an enzyme involved in differentiation, is regulated almost entirely post-translationally in shoots by localised fluxes of calcium and by compartmentation of calcium and calmodulin in relation to the compartmentation of the enzyme. The functions for which the resulting coenzymes are employed may vary accordingly. The enzyme may be similarly regulated in root tissues. Furthermore, the alteration in ratio and concentrations of NAD and NADP resulting from differential NAD kinase activity may regulate activity of other enzymes involved in differentiation in a post-translational manner, further indicating that enzyme concentration is not the only factor regulating activity; and that non-protein compounds may be involved in regulation of enzymes associated with development.

It is evident from examination of total and individual protein species in the root apex that protein composition develops along different and less uniform patterns from those specified by the model of Brown. The range of mechanisms by which proteins control differentiation also appears to be far broader than envisaged by the model.

The control of protein composition will be considered in the following section.

C. Developmental changes in, and control of, protein synthesis and composition

(i) Transcriptional control during root differentiation

Brown regarded protein synthesis, and eventually protein concentration and enzyme activity, as being under transcriptional control. A progressive alteration in transcription was envisaged as occurring *via* a sequential production of mRNA regulators during development. As discussed in chapters 1 and 4, only indirect evidence for this hypothesis existed at the time of writing; and although it is still generally accepted that control of protein synthesis in the root apex is under transcriptional control, no further evidence for this has been obtained.

Although protein concentration per cell did not alter substantially throughout the root apex, marked change in protein composition appeared to occur during differentiation as analysed by two-dimensional PAGE and by biological activity of individual proteins. However, although it appeared from electrophoretic analysis of (^{35}S) methionine labelled proteins that many of the changes in protein composition were accompanied by corresponding changes in protein synthesis, this did not occur in all cases, indicating that some post-transcriptional control of protein concentration may occur. However, many of the changes in protein composition did appear to be accompanied by a similar change in translation, and the possibility that this reflected transcriptional control was investigated.

It was observed that while protein synthesis decreased per cell to an extremely low level by 10mm from the apex, both rRNA and *in vitro* translatable mRNA rose per cell, indicating indirectly that an increasing proportion of RNA becomes redundant during cell differentiation and maturation. Protein synthesis therefore appeared to become increasingly limited at the post-transcriptional level. As relatively little change in *in vitro* translation products appeared to occur between different stages of development, the change in *in vitro* translatable mRNA appeared to be largely non-specific. The relative lack of detectable change in *in vitro* translation products was in marked contrast to the extensive

changes occurring in protein composition and synthesis. Despite the fact that many mRNA species will not have been detected by this procedure and that the concentration of some proteins may be under transcriptional regulation as several mRNA species did alter in concentration throughout development, the contrast in *degree* of change in protein synthesis and composition with that of *in vitro* translatable mRNA was striking, further supporting the proposition that control of protein synthesis is predominantly at the post-transcriptional level. This is in direct contrast to Brown's hypothesis and to the currently accepted view that control of protein synthesis in the root apex is predominantly transcriptional.

(ii) Post-transcriptional control of protein concentration and protein composition

As indicated in chapter 4, apparent specific activity of protein synthesis per cell as estimated by (^{35}S) methionine incorporation into protein fell rapidly during differentiation indicating indirectly that the average turnover rate of protein altered during differentiation. This fall occurred earlier in the stele than the cortex, at 2-4mm from the apex during the main region of sieve tube maturation. This may be partly due to the breakdown of the nucleus in these cells: unless long-lived mRNA coded for by the nucleus were present, concurrent protein synthesis in sieve tubes would be directed largely by plastid and mitochondrial genomes, utilising largely organellar protein synthesising machinery. A qualitative as well as a quantitative change in protein synthesis should therefore occur in these cells. As the stele occupied an increasing proportion of total protein in the root, due to a slower decline in protein in the stele than the cortex, concurrently with the apparent fall in specific activity of protein synthesis, it is possible that this also reflects an increasing proportion of protein with a low turnover rate. This may include structural protein in the secondary wall and protein involved in filament formation in sieve tubes. It may also reflect a lower rate of vacuole enlargement in xylem and non-sieve tube phloem cells compared with that of cortical cells. By contrast, the region of highest apparent specific

activity of protein synthesis in the cortex coincided not only with the region of the rapid fall in specific activity of protein synthesis in the stele, but also with the region of most active vacuolar autophagy, and it seems possible that vacuolar autophagy may act as a mechanism for rapid turnover of metabolites for novel protein synthesis.

Two-dimensional electrophoresis indicated that differential degradation of specific proteins might be involved to some extent in alteration of protein composition as well as altering total concentration. As controlled cell breakdown appeared to be a major feature of differentiation, a mechanism of post-translational control could involve the regulation of timing and selectivity of protein degradation. Vacuoles may have a prominent role in this process, either through release of lytic enzymes as may occur in xylem and root cap cells, or by autophagy. Autophagy may be obviously selective as in metaphloem sieve elements, possibly allowing the selective retention of plastids, mitochrondria and E.R., or may be non-selective as in cortical, epidermal, pericycle, and endodermal cells. Non-selective autophagy may not only regulate total protein concentration in the cell, but may permit rapid alteration of protein composition through rapid sequestration of protein. Regulation of tonoplast properties may therefore also be involved in regulation of differentiation.

Most of the alteration in protein composition during differentiation appeared to be regulated at the level of protein synthesis. It is possible that although the composition of mRNA translatable in the wheatgerm S30 *in vitro* system did not appear to alter substantially during development, that release of specific species of mRNA to the cytoplasm may be involved in specificity of translation.

The composition of RNA, consisting largely of rRNA, has however, been found to vary during root development (Heyes, 1960). The composition of rRNA might be regulated at the transcriptional level. However there is no evidence to support this; nor is there evidence to indicate that transcriptional control of rRNA, if it occurs, is regulated by specific protein regulators of transcription as proposed by Brown. Ca^{2+} ATPase activity associated with the nucleolus has been observed to decrease as

cells mature (Sexton and Sutcliffe, 1969), and a corresponding change in the nucleolar ionic environment might therefore be expected. This might affect transcription by alteration of rDNA condensation. A number of other nucleolar activities might also be affected, including rRNA aggregation and ribosome assembly.

It has also been noted that the ultrastructural characteristics of the nucleolus alter as cells mature, indicating that incomplete ribosomal precursors accumulate in nucleoli of maturing cells possibly due to a shortage of one or more precursors (Chaly and Setterfield, 1975). These observations indicate that an alteration in composition or in availability of ribosomal proteins and/or rRNA may occur during differentiation. A nucleolus-associated body, the karyosome, has been implicated in the transfer of ribonucleoprotein from the nucleolus to the cytoplasm (Barlow, 1983; Williams *et al.*, 1983). It has been observed to be more frequent in differentiating and maturing cells than dividing cells (Barlow, 1983). Selective or quantitative transport of ribosomal precursors could possibly be mediated at least in part by karyosomes, either through selective or quantitative release of ribosomal precursors to the cytoplasm, or by selective transport of specific precursors into or out of the nucleolus.

The transport, synthesis, and/or assembly of ribosomal precursors may therefore alter ribosome composition during differentiation. This may alter specificity of translation directly by affecting translation of specific mRNA species, or may alter it indirectly through affecting the relative efficiencies of initiation of specific mRNA species. Alternatively, a non-specific alteration in capacity for protein synthesis may result from alteration in ribosome composition, but this will also differentially affect protein synthesis with respect to mRNA species that have different efficiencies for initiation or elongation.

Both quantitative and qualitative changes in protein composition may therefore be achieved by specific controls, or by non-specific changes acting on a population of genes, mRNA species, or proteins that are heterogeneous with respect to such

characteristics as rate of turnover and efficiency of various aspects of transcription and translation.

In contrast to the proposal of Brown that protein composition and enzyme activity are entirely under transcriptional control, very little evidence for such extensive transcriptional control was obtained. The predominant levels of control in regulation of protein synthesis and composition appeared to be post-transcriptional and post-translational. Post-translational control mechanisms also appeared to regulate the activity of a variety of enzymes and proteins involved in differentiation, as discussed in section 3 B.

D. Summary

The patterns of changes in protein composition and enzyme activity during differentiation, and their control, clearly differed in a number of ways from the model of control of gene expression during root development of Brown (1963, 1964). The manner of protein control of differentiation also appeared to differ from that specified by the model.

Briefly, the main areas of disagreement with the model include

1. Lack of uniformity in development of protein patterns in different tissues as specified by the model. Changes in levels of proteins do not occur at the same time and in the same direction in all cells at the same transverse level.
2. Not all proteins increase during cell expansion, reach a peak, and then stabilise or decline.
3. Tissue-specific and stage-specific proteins were detected.
4. The activities of enzymes involved in differentiation may be regulated not only by concentration, but also by protein activators.
5. Enzyme activity may also be regulated by a variety of post-translational control mechanisms involving non-protein regulation, specifically including levels of ionised calcium, coenzyme levels, compartmentation, and degradation.
6. Enzymes are not the only forms of protein that may be

involved in differentiation. Activator, contractile, and structural protein may also be involved. These proteins may also be regulated at the post-translational level by regulation of activity, assembly, polymerisation, compartmentation, intracellular movement, and degradation.

7. Changes in concentration of an activator protein may have a functional significance in the regulation of differentiation.

8. The functions of an activator protein may vary throughout differentiation.

9. Transcriptional control of changes in protein synthesis appeared to be minimal. Most control of gene expression appeared to be at the post-transcriptional including post-translational levels.

Control of gene expression is therefore far more complex than indicated by the model, involving less uniformity of trends and a far greater range of control mechanisms. The results from both biochemical and structural studies have a number of implications for the control of differentiation, and these will be discussed in section 4.

4. THE CONTROL OF DIFFERENTIATION IN THE ROOT APEX

A. Comparison of the control of gene expression during differentiation in the root apex with the mechanisms of control in other species

Epigenetic theories of differentiation tend to concentrate on transcriptional control mechanisms, and it is currently accepted that protein synthesis in the root apex is probably largely if not entirely under control at the transcriptional level. As described above, however, transcriptional control in the pea root apex appeared to be minimal as differentiation progressed. Regulation of gene expression during early cell differentiation in the root apex appeared to be predominantly at the post-transcriptional level.

This is in contrast to the apparent transcriptional control of abundant tissue-specific proteins which occurs in mature cells and in a variety of developmental events in both plant and animal cells as described in chapter 1, and also to control of mammalian embryogenesis in which transcriptional control may be detected at an early stage (Davidson, 1976). Although post-transcriptional control does occur in early stages of morphogenesis in a number of lower organisms as described in chapter 1, these systems are not strictly comparable to post-transcriptional control in the root apex, as differential mRNA accumulation may occur either within a single cell, as in *Acetabularia*, or between cells as a result of polarisation with subsequent division as in oogenesis of the sea urchin. Post-transcriptional regulation of single-celled algae and fungi may also be dissimilar, as transcription of specific mRNA species associated with early morphogenesis may well occur prior to the event; and although early morphogenesis may not require concurrent transcription, specific mRNA species may already be present. In all these cases, although concurrent mRNA transcription is not required, differential mRNA accumulation is involved. There is no evidence to suggest that this may occur in the root apex. Post-translational control of initial events of germination in *Blastocladiella emersonii* zoospores apparently under control of a burst of intracellular ionised calcium (Soll and Sonneborn,

1969, 1972; Hutchinson *et al.*, 1977; van Brunt and Harold, 1980) as occurs in a number of species in germination (Gilkey *et al.*, 1978; Berridge, 1976; Steinhardt and Epel, 1974), may be regarded as de-differentiation or termination of a resting state rather than initiation of differentiation. Again this is not strictly parallel to the situation in the root apex.

B. The significance of post-transcriptional control for differentiation in the root apex

As described in chapter 1, differentiation in plant cells is remarkably plastic, whereas differentiation in animal cells is generally irreversible. The latter may be a reflection of relatively permanent or stable changes in DNA structure (Wang *et al.*, 1979) which may irreversibly regulate transcription either, for example, through specific alteration of the three-dimensional structure of DNA possibly involving transcription enhancers (Benoist and Chambon, 1981; Gruss *et al.*, 1981; Nordheim and Rich, 1983), or through intramolecular movement of sections of DNA (Steffensen, 1977).

Transcriptional control generally appears to be associated with organisms undergoing strictly regulated and relatively inflexible differentiation; or with cells that are required to respond in an inflexible manner to inductive stimuli in order to maintain a functional organism. By contrast, many lower organisms appear to have early morphogenesis regulated post-transcriptionally. This appears to be associated with organisms that require either flexibility in the pattern of differentiation and a rapid response to stimuli inducing change, as with early cell differentiation in the root apex; or that require flexibility in timing of commencement of morphogenesis and a rapid response to stimuli inducing morphogenesis, as in the cases cited above which are associated with prior differential mRNA accumulation.

The apparently low occurrence of transcriptional control of protein synthesis, and presumably therefore the similarity in capacity of the genome for transcription in different cell types during early differentiation in the pea root apex, may therefore reflect the more flexible nature of plant cell differentiation. As plant cells rapidly have to adjust to wounds inflicted by animals or by the environment, and to changing environmental

conditions, such flexibility both in terms of the capacity for re-organisation of tissue patterns and the ease with which this may be accomplished may have a functional value.

C. Changes in metabolic state in the root apex as specified by Brown (1963)

(i) The meristem and specification of differentiation

Brown developed the model of the molecular basis of differentiation at the root apex on the basis that cells in the meristem were a mass of homogeneous uncommitted cells that were induced to change state to an expanding, differentiating state by means of an unequal cell division at the base of the meristem. The dividing cell was held to be incapable of differentiation by virtue of its enzymatic state.

Indeed it is now widely accepted that differentiation is specified within the apex itself as a result of positional controls (Sachs, 1978; Foard, 1959) or through the properties of the apex itself (Torrey, 1955). This contrasts with a former view of differentiation in which, as it was noted that specific apical initials gave rise to different tissues (Hanstein, 1868; Janczewski, 1874), differentiation within a cell lineage was regarded as being specified by the apical initials with original specification considered to take place in the embryo proper.

However, there is evidence to support the proposition that differentiation has already taken place by the level of the quiescent centre as anatomical differences between tissues may be traced into the quiescent centre (Torrey and Zobel, 1977; Popham, 1955; Feldman and Torrey, 1976). However, cytological studies concentrate on differences in cell division and cell shape characteristics between cells, and thus it might be argued that differences observed between putative tissues in the meristem are not an expression of differentiation, but rather reflect differences in localised concentrations of cell division factors. Perhaps as a result of this consideration, the different meristematic areas of procambium, procortex, and protoderm are commonly regarded, despite structural differences, as unspecified groups of meristematic initials in which differentiation may be induced. However, as discussed in

chapter 4, section 1, ultrastructural as well as anatomical differences may be detected even at the distal end of the meristem in the pea root apex.

There is also evidence to support the proposition that commitment has occurred by the level of the quiescent centre. Extremely small pieces of tissue including the quiescent centre (Torrey, 1955; Reinhard, 1955), or the quiescent centre alone (Feldman and Torrey, 1976) may be isolated and may reform the root apex directly without an intervening callus stage. Furthermore, application of auxin to roots results in differential effects on the polarity of division in different cell types at an extremely early stage in the meristem (Burström and Svensson, 1974), indicating that the response to cell division factors depends on the inherent potential of the cell, and that cells are already committed at a very early stage in the meristem.

Although the capacity for re-organisation of the apex is generally regarded as demonstrating the absence of a pre-determined state in the meristem, the ease of reversibility of differentiation and of the induction of new developmental pathways in differentiating or maturing cells indicates that it is possible for a cell to be adaptable even if progressing, or possibly specified to progress, along a particular pathway. Re-organisation of tissue patterns at the apex may therefore represent re-induction of differentiation rather than an initial lack of specification. Control of specification of the major tissue types may therefore originally occur within the embryo with subsequent differentiation dependent on cell lineage within the root apex. Although it appears that a further range of tissues may be induced to mature from procambial cells with the pattern dependent on concurrent conditions in the meristem, this may be dependent on cell lineage patterns, and the procambium may therefore be considered either as a completely determined tissue, with tissue patterns dependent on the pattern of cell division, or as a partially pre-determined tissue from which a limited range of cells may develop at an early stage in the meristem, rather than as a completely unspecified mass of cells.

The meristem therefore does not consist of a mass of homogeneous cells as claimed by Brown. Nor is the dividing state

incompatible with differentiation. It is also unlikely that differentiation occurs as late as the base of the meristem as proposed by Brown, and indeed the assumption that the apex is a region of primary specification of differentiation may not even be valid.

Specification of differentiation is generally envisaged as involving control of blocks of genes (Jacob and Monod, 1961; Monod and Jacob, 1961; Meins, 1975). However, although the specification stage of differentiation and the control of transcription in different cell types were not examined in pea root, transcriptional control of mRNA appeared to be minimal in the pea root apex as differentiation progressed. It therefore seems possible that specification may either involve largely post-transcriptional control mechanisms; transcriptional regulation of a small number of genes; transcriptional control of genes that code for very minor proteins; or transcriptional control for a very brief period.

(ii) The growth phase in the root apex, and the expression phase of differentiation

Brown proposed that cell development in the root apex involved a succession of metabolic states. Cells in the initial unspecified meristematic state were considered to possess a metabolic (enzymatic) system in which growth could not occur. Through development of an appropriate metabolic (enzymatic) system, growth was permitted. Differentiation became apparent through, and because of, cell growth, as a result of differential growth from initially unequal cell complements. The metabolic states in the meristematic and expanding cells were held to permit qualitatively different activities.

As described in chapter 4 section 1, polarity and rate of growth appeared to vary between, and to be highly characteristic of, individual cell types. Thus growth appeared to be an integral part of differentiation and regulated by cell type. However, it is arguable that differentiation can only become apparent through growth as represented by an increase in dry weight as proposed by Brown. Alteration in composition of cytoplasm, cell wall material, and extracellular secretions may

presumably occur without such an increase, while cytoplasmic degeneration in contrast to growth is an integral part of differentiation of some cell types. Furthermore, division characteristics are dependent on tissue, and this may be observed at the extreme apex of the root, long before the so-called 'zone of expansion' commences. Thus differentiation may not necessarily require cell growth for its expression. Moreover, contrary to the traditional view, cells do not in fact undergo a qualitative change in activity from a dividing to an expanding differentiating state. Growth and differentiation both occur within the meristem; specific regions remain meristematic during cell maturation; and radial expansion continues throughout at least the apical 20mm. Although an alteration in rate of expansion between the apical meristem and the subjacent zone of rapid elongation does occur, this difference is quantitative and directional.

Considerable cell expansion occurs within the apical meristem of pea root, cell volume increasing 2-7-fold within 500μ of the quiescent centre/root cap junction depending on cell type, despite these cells having undergone several divisions within this region. This is due largely to radial and tangential expansion. Although cell divisions are predominantly transverse, thus reducing apparent cell elongation within the meristem, there is a decrease in radial expansion and an increase in relative rate of elongation at the base of the meristem. A change from predominantly radial cell expansion to predominantly longitudinal cell expansion therefore accompanies the increase in relative rate of elongation.

An alteration in the rate of elongation at the base of the meristem has long been recognised and it has often been suggested that cells in the apical meristem cannot or do not grow, that cell division and expansion are mutually exclusive processes, that they are relatively independent (Brown, 1963; Dormer, 1972) or that there is a conflicting interest between cell division and cell growth (Clowes, 1961). As division and growth are not in fact mutually exclusive in the root apex, the latter interpretation may be more accurate. However, this does not necessarily imply a change in concentration and activity of certain enzymes as proposed by Brown.

As a change in both polarity and rate of growth occur at

the base of the meristem, it is possible that alteration in availability and orientation of existing factors involved in expansion occurs. This may include the involvement of structural proteins. Alternatively, differences in the capacity to grow may reflect differences in type and distribution of growth substances or growth substance receptors; differences in capacity for expansion, for example for formation of osmotically-active solutes or synthesis of cytoplasmic material; to different availability of growth factors, nutrients or growth inhibitors. A difference in capacity for growth does not necessarily involve a difference in enzymatic states.

Three main models have been proposed for the control of expansion in the root apex. Firstly the proposal of different enzymatic states of Brown. Secondly, differential distribution and concentration of growth factors. Thirdly, the alteration in rate of expansion has been attributed to competition between the requirements of cell division and expansion for microtubules and microtubule organising centres (Trewavas, 1982; Lloyd and Barlow, 1982). During division and expansion, microtubules are involved in several processes (Lloyd and Barlow, 1982; Gunning and Hardham, 1982) including the regulation of cell expansion during interphase; formation of the preprophase band which anticipates the plane of cell division; structure and regulated assembly and disassembly of the mitotic spindle and formation of the phragmoplast in which microtubules help guide wall-forming vesicles to the site at the cell plate at which they coalesce to divide the forming daughter cells. Microtubules are required for continued deposition of cellulose microfibrils in the cell wall during cell expansion. Competition for available tubulin subunits between these processes might clearly limit the rate of expansion during cell division, and their orientation during microfibril deposition may influence cell shape and future potential for polarity of expansion. Thus calmodulin may be involved in regulation of the rate and polarity of expansion and the differential rates of elongation in meristematic and elongating areas through regulation of microtubule polymerisation and localisation.

Calcium has also been implicated more directly in regulation

of the rate and polarity of expansion (Moll and Jones, 1981; Stevenson and Cleland, 1981), possibly through an effect on auxin-induced cell wall expansion (Coartney and Morré, 1980; Roux and Slocum, 1982; Marré *et al.*, 1974; Cleland and Rayle, 1977). As calcium has also been implicated in polarity of growth through localised electrophoretic effects resulting from a cellular gradient of calcium which occurs in growing tips (Herth, 1978; Reiss and Herth, 1979a, 1979b; Meindl, 1982; Jaffe *et al.*, 1974, 1975; Weisenseel *et al.*, 1979; Robinson and Jaffe, 1975; Saunders and Hepler, 1981), it is possible that calmodulin may also regulate the rate and polarity of cell expansion by regulation of Ca^{2+} ATPase activity and thus localised calcium concentration and distribution. However, although this involves enzyme activity, the distribution of Ca^{2+} ATPase in roots alters from association with the nucleus in meristematic cells, to the plasmalemma in expanding cells (Sutcliffe and Sexton, 1969). Thus the distribution rather than the concentration of Ca^{2+}-ATPase may be important in regulation of activity.

There is also evidence to support the proposition that the involvement of calmodulin in geotropism (Biro *et al.*, 1982; Chandra *et al.*, 1982; Roux and Slocum, 1982) acts *via* calmodulin regulation of calcium efflux into the cell wall through regulation of Ca^{2+} ATPase activity. The redistribution of calcium (Slocum and Roux, 1983; Goswami and Audus, 1976), may cause a reduction in rate of growth in areas of high calcium concentration, with a consequent bending response (Digby and Firn, 1979; Carrington and Firn, 1983).

As mentioned above the alteration in rate of expansion has also been attributed to hormonal control *via* auxin effects on cell wall 'loosening' (Torrey, 1953) through stimulation of H^+ secretion and synthesis of macromolecules for growth (Rayle and Cleland, 1977; Trewavas, 1976). However, as almost all auxin in roots is confined to the stele (Greenwood *et al.*, 1973), such an asymmetric distribution would seem unlikely to regulate rate of expansion, although it may act as a permissive rather than a regulatory force.

It is clear from these considerations that alteration in capacity for growth beyond the meristem need not reflect changing

enzyme concentrations and activities, but rather distribution and concentration of activator proteins, structural proteins and ionised calcium, and the distribution as well as the concentration of enzymes.

It was noted that, in addition to a general change in capacity for growth at the base of the meristem, the capacity for growth also differed between cell types within the 'zone of expansion'. It was also observed that during radial expansion of the root, there was not a radial gradient that permitted a coordinated gradient of growth rate in different tissues. Different cell layers grew differentially not only in rate but also in polarity, and this was reflected in the differences in change of cell shape and size, the proportion of a tissue occupied by intercellular spaces, and in the proportion of the root occupied by different tissues (chapter 4 section 1 B ii b). The manner of compensation through growth and change in shape for stress imposed by adjacent cell layers also appeared to depend on the inherent capacity of the cell to respond. Furthermore, Burström and Svensson (1974) have noted that auxin differentially affected growth in different cell types, increasing growth predominantly in cortical cells. Burström (1971) has also noted that the root is under longitudinal stress due to a relatively higher growth rate in the cortex than inner layers. As it was noted that in normal root development (chapter 4 section 1 B) most radial expansion beyond the meristem occurred in cortical cells, the reaction of cells to auxin further supports the proposition that the capacity of cells to respond is the predominant factor in the response to growth factors.

This difference in polarity and rate of growth in expanding cells beyond the meristem may reflect differential capacity for rate of growth in combination with restrictions in the direction of growth. Restriction of growth might arise from rigidity of the cell wall. This may vary in different cell types due to differential laying down and alignment of cellulose microfibrils during interphase in cells in the apical meristem and thus affect both polarity and rate of growth. Restriction of growth may also reflect such factors as Ca^{2+} ATPase activity and distribution as this enzyme varies in concentration between different cell

types in expanding cells (Sutcliffe and Sexton, 1969). Restriction in the rate of growth may also reflect such factors as capacity to form osmotically active solutes which may differ between cell types, possibly being high in the cortex due to autophagy during the period of cell growth; and it may also reflect cytoplasmic degeneration in protophloem and metaphloem sieve tubes which might reduce the capacity for expansion of the stele. Again these growth-regulating mechanisms reflect largely post-transcriptional control, and emphasise the significance that non-enzyme compounds may have in control of growth and differentiation.

Whether specification of differentiation is regulated predominantly at the transcriptional or post-transcriptional level, it appears from analysis of *in vitro* translation products, protein synthesis, and protein composition, that the expression phase of differentiation, including growth, is regulated largely at the post-transcriptional level. This is inconsistent with the proposition of Brown that differentiation is regulated at the transcriptional level. Furthermore, the hypothesis that cell development involves a succession of enzymatic states from one permitting a non-expanding, meristematic, non-differentiating state to an enzymatic state permitting expansion and differentiation but not division, is clearly not tenable on structural grounds, and appears to be too simplistic a description of biochemical events involved in division growth and differentiation to be a useful model of the control of differentiation in the root apex.

D. Summary

Cell development in the pea root apex does not appear to be under control of sequential enzymatic states permitting qualitatively different processes as envisaged by Brown (1963); but rather the processes of division, expansion, and differentiation are intimately related at the structural, ultrastructural, biochemical and functional levels. Furthermore, there appears to be a remarkable lack of coordination in growth and differentiation between tissues, and it appears that growth and differentiation take place on a cellular or tissue level. Division characteristics

and the rate of cell differentiation are also highly characteristic of cell type. Uniform anatomical and biochemical trends therefore do not occur between different cell types, and the root does not appear to develop as a unit with strictly regulated longitudinal zones of coordinated activities.

Gene expression during differentiation similarly does not appear to undergo such coordinated overall trends as described by Brown. Each tissue appears to undergo a unique pattern of protein synthesis, breakdown, and final concentration. Again in contrast to the specifications of the model, cell growth and maturation in the root apex appear to be controlled largely at the post-transcriptional level, although the possibilities that specification and/or fundamental control of cell maturation are under transcriptional control are not excluded. Molecular control of development does not therefore appear to consist predominantly of sequential synthesis of regulators of transcription, with similar patterns of protein synthesis in different cell types at any transverse zone. Furthermore, control of enzyme activity is not regulated predominantly at the transcriptional level, nor are enzymes the only form of protein involved in differentiation. The pattern of biochemical development during differentiation appeared to be far more complex both in terms of differences between tissues and in the range of control mechanisms than indicated by the model.

Again in contrast to the model, cell differentiation appears to be predetermined either at an extremely early stage in the meristem, or by apical initials whose developmental fate is specified in the embryo. Thereafter, differentiation appears to be relatively autonomous, and division, growth, and differentiation proceed at a rate and in a manner characteristic of each tissue at the ultrastructural, biochemical and functional levels. Although many characteristics develop progressively in a similar manner in different tissues, many do not. Differentiation at the structural, ultrastructural, biochemical and functional levels are far more complex than envisaged in the model of root development of Brown.

5. FUTURE WORK

A. The role of calmodulin in root development

Calmodulin is clearly potentially involved in a wide range of functions concerned with differentiation and development in the root apex, with regulatory activity altering throughout differentiation. However, as indicated previously, activity and function are dependent on the localised concentrations and distributions of calmodulin in relation to those of calmodulin-binding proteins and ionised calcium. In order to assess the *in vivo* functions of calmodulin more realistically, and to evaluate its relevance to differentiation in the root apex, it will therefore be necessary to localise these factors and to estimate their concentration in order to obtain an indication of whether their localised concentrations are compatible with activity.

Two main areas have been indicated from this study as being of particular interest, and worthwhile pursuing further. One of these areas involves the role of calmodulin in cytoskeletal events through control of polarity of cell division and expansion, and therefore indirectly of histogenesis, morphogenesis and maintenance of root shape and structure, and also through control of rate of growth in meristematic areas. The other area involves the role of calmodulin in carbohydrate metabolism through regulation of the concentrations and ratio of NAD to NADP through activation of calmodulin-dependent NAD kinase.

(i) Regulation of microtubule activity

Calmodulin regulates several processes in division and expansion. The regulation of microtubule polymerisation and of localised calcium concentration are particularly interesting as these have a central role in both polarity of growth and division, and in rate of growth.

As discussed in section 3 A i, calmodulin may control a number of microtubule activities either *via* direct control of microtubule assembly/disassembly through binding to or phosphorylation of tubulin or microtubule associated proteins; or

indirectly through localised regulation of calcium concentration by regulation of Ca^{2+} ATPase activity. However, to further investigate the potential role of calmodulin in the control of the rate and polarity of growth and division, it will be necessary to determine which microtubule activities are regulated by calmodulin, whether different sub-populations of plant microtubules are differentially affected by calmodulin and what the mechanisms of regulation are.

Initial experiments could include the use of immunoelectron microscopy to localise calmodulin in root cells, followed by quantitation in different cell fractions. Developmental changes in calmodulin-binding proteins could then be examined by purification of calmodulin-binding proteins by affinity chromatography and analysis by polyacrylamide gel electrophoresis. Tubulin, microtubule associated proteins and Ca^{2+} ATPase could be identified by immunoblotting. As different populations of tubulin and microtubules are known to exist (Hyams, 1982), and may be differentially transcribed during embryogenesis in *Drosophila* (Raff *et al.*, 1982), while cold-stable microtubules are known to be particularly sensitive to calmodulin, the sensitivity of different tubulin subunits to calmodulin-inhibition of polymerisation could be examined. If differential sensitivity were observed, antibodies to the different subunits could be raised and utilised for immunoelectron microscopy. Similarly, differential calmodulin-sensitivity of microtubule depolymerisation could also be examined, and antibodies specific to the calmodulin-sensitive microtubules could perhaps be raised. Intracellular localisation of calmodulin-sensitive tubulin and microtubules during division and expansion in the root apex could then be compared with that of cold-stable microtubules and the total microtubule and tubulin population in order to assess which microtubule activities may be regulated by calmodulin. In order to then assess whether calcium distribution may regulate calmodulin-dependent microtubule polymerisation, calcium could be located by the antimonate/tannic acid precipitation technique, with energy dispersive X-ray microanalysis of the precipitates (Slocum and Roux, 1982). As free calcium in tissue sections is very hard to estimate however, activated calmodulin-dependent

sites could also be examined indirectly *via* localisation of unbound calmodulin sites through the use of a fluorescent calmodulin probe (Pardue *et al.*, 1981; Dedman *et al.*, 1982) and comparison of these with total and calmodulin-sensitive tubulin and microtubule distribution.

Determination of the concentrations and proportions of different populations of microtubules and microtubule associated proteins, could then be examined in different regions of the root apex to determine whether a changing population of microtubules or microtubule associated proteins may occur concurrently with changing microtubule function.

The possible control of Ca^{2+} ATPase by calmodulin could also be investigated, first by enzyme assay to establish whether a calmodulin-independent Ca^{2+} ATPase is present in addition to a calmodulin-dependent isozyme. Intracellular localisation by immunoelectron microscopy could then be carried out, and compared to the distribution of calmodulin, tubulin, microtubules, calcium, and unbound calmodulin-binding sites. Analysis of the concentration of calmodulin-dependent ATPase in different cell fractions throughout the root could then be carried out and compared with localisation by ultrastructural studies.

Finally, the possibility that calcium-binding proteins other than calmodulin exist in plant cells and may have a role in development in the root apex, could be investigated by incubation of Western transfers of polyacrylamide gels or of non-denaturing gels with $^{45}Ca^{2+}$, or with the carbocyanine dye 'Stains all', (Campbell *et al.*, 1983).

(ii) Regulation of NAD kinase activity

The other area of calmodulin regulation that it would be of interest to pursue involves the regulation of carbohydrate metabolism by pyridine nucleotide levels and ratios through regulation of the activity and compartmentation of NAD kinase. Although total cellular concentration of calmodulin did not limit NAD kinase activity in crude homogenates of root tissues in the presence of micromolar levels of calcium, the intracellular distribution of these proteins and of free calcium in the root is not known. It is therefore not possible to assess

whether the apparent *in vivo* increase in NAD kinase activity (as an increase in the NADP:NAD ratio) which occurs concurrently with the *in vitro* increase in NAD kinase activity is due to an increase in ionised calcium in the relevant compartments, to an increase in localised calmodulin concentration if calmodulin rather than calcium were present in limiting concentration, or to an alteration in compartmentation of either of the proteins. In order to evaluate whether the changing levels of the calmodulin-dependent NAD kinase may be functional *in vivo*, and to obtain an indication as to the function of the altering NAD:NADP ratio during differentiation, the intracellular distribution of the two putative isozymes should be investigated, and the regulation *in vivo* of the calmodulin-dependent form investigated by comparison of its localised concentration with that of calmodulin and free calcium.

In view of the apparent alteration in ratio of the two forms of activity, and the possibility of difference in compartmentation, it is of importance to establish whether the two forms of NAD kinase activity represent different isozymes, or whether one of the forms is extract-generated. If two isozymes do in fact exist, the changing ratio of an apparently non-regulated isozyme to a rapidly regulatable isozyme might have a developmental signficance. As discussed in chapter 3 section 3 there is indirect evidence to suggest that the forms are not extract-generated; however, this could be further investigated by purification of the isozymes to homogeneity. The purified proteins could then be analysed by amino acid sequencing, and the effect of the extraction procedure on enzyme activity assessed by comparison of the activities and calmodulin-dependence of activity of the purified proteins assayed alone and after extraction with a sample of pea root tissue. If two isozymes are in fact present *in vivo*, further characterisation of the purified protein would yield valuable information for the assessment of their potential activity and regulation *in vivo*.

Compartmentation of the isozymes could be investigated by immunoelectron microscopy, and additionally quantitated following cell fractionation. Comparison of the localisation and concentration of the calmodulin-dependent NAD kinase could be

compared to that of calmodulin. Calcium distribution could be observed by the antimonate/tannic acid precipitation method with X-ray microanalysis of the precipitates. As for tubulin and Ca^{2+} ATPase, the localisation of antibodies to the calmodulin-dependent NAD kinase could be compared with fluorescent calmodulin to estimate the presence of unbound, and thus probably inactive, calmodulin-dependent NAD kinase. Analysis of regulation of calcium fluxes within the relevant compartments could be carried out by isolation of different cell fractions including plastids and plasma membrane fractions, and by observation of the effect on uptake and efflux of calcium by plant growth substances, cations, phytochrome and calcium. Kinetic parameters of calcium uptake and efflux could be studied by the use of $^{45}Ca^{2+}$, and by fluorimetry involving Quin 2 which binds to calcium in stoichiometric quantities (Tsien, 1980; Tsien *et al.*, 1982; Rink *et al.*, 1982), aequorin which fluoresces in the presence of free calcium (Cormier *et al.*, 1980), and chlorotetracycline which will fluoresce in the presence of membrane-bound calcium. Protoplasts could additionally be investigated by including the use of murexide, which measures changes in concentration in extracellular calcium as low as 1μM.

Of particular interest is the regulation of calmodulin-dependent NAD kinase by phytochrome. As indicated previously, NAD kinase in shoot tissue may be regulated both *in vivo* and *in vitro* by phytochrome, and it is possible therefore that phytochrome exerts an effect on NAD kinase activity through direct activation or release from membranes rather than, or in addition to, regulations of calcium fluxes. The effect of phytochrome on both forms of purified NAD kinase, and on NAD kinase in crude extracts and *in vivo* could therefore be studied in conjunction with the influence of phytochrome on calcium distribution.

Should NAD kinase activity be observed to be present in different cell compartments, the control of the distribution of NAD kinase could be initially investigated by examining the presence of the mRNA(s) and structural gene(s) for NAD kinase in different cell compartments.

These experiments should provide a preliminary indication of the relative contributions *in vivo* of different forms of NAD

kinase; differential regulation of NAD kinase activities; the potential role of calmodulin in regulation of pyridine nucleotides and the potential significance of the changing levels of pyridine nucleotides through compartmentation.

B. Transcriptional control of protein synthesis

A further major area of interest in this study is the apparent lack of extensive control of protein synthesis at the transcriptional level as estimated by *in vitro* translation. This should be studied further, and initial experiments could include the translation of mRNA using different *in vitro* translation systems in order to establish whether the wheatgerm systems selectively translated specific pea mRNA species or affected translatability artifactually. An *E.coli* system could be used to translate plastid mRNA. The relative abundance of mRNA species between different tissues could also be examined, as it is possible that greater differences will be found between rather than within tissues during development. Polysomal mRNA should also be translated and compared to total mRNA translation products in order to obtain an indication as to whether control of protein synthesis may occur predominantly at the pre-translational or translational level. The composition of ribosomes during differentiation, and the effect of ribosomes from different zones of the root apex on *in vitro* translation products could also be investigated in order to assess whether changes in ribosomes could affect translation *in vivo*.

It is possible that the results obtained for relative abundance of mRNA were a consequence of an artifact of translatability of mRNA *in vitro*. It is therefore desirable to use another method of analysis. Preliminary experiments could include synthesis of cDNA obtained to the mRNA of one zone in the root apex, and its hybridisation to mRNA of other zones. The unhybridised mRNA could then be translated *in vitro* and compared to *in vitro* translation products of the mRNA that hybridised to the cDNA probe, in order to distinguish between quantitative and qualitative changes in mRNA. These experiments could be repeated with cDNA to individual mRNA species for more accurate analysis.

REFERENCES

Abelson, J. (1979) Ann.Rev.Biochem. *48*, 1035-1069.

Adelman, T.G. and Lovett, J.S. (1974) Biochim.Biophys.Acta *335*, 236-245.

Ames, G.F.-L. and Nikaido, K. (1976) Biochem. *15*, 616-623.

Ames, G.F.-L., Spudich, E.N. and Nikaido, H. (1974) J.Bacteriol. *117*, 406-416.

Amphlett, G.W., Vanaman, T.C. and Perry, S.V. (1976) FEBS Lett. *72*, 163-168.

Andersen, B., Osborn, M. and Weber, K. (1978) Cytobiologie *17*, 354-364.

Anderson, J.M., Charbonneau, H., Jones, H.P., McCann, R.O. and Cormier, M.J. (1980) Biochem. *19*, 3113-3120.

Anderson, J.M. and Cormier, M.J. (1978) Biochem.Biophys.Res.Commun. *84*, 595-602.

Apel, K. (1979) Eur.J.Biochem. *97*, 183-188.

Apel, K. and Kloppstech, K. (1978) Eur.J.Biochem., *85*, 581-588.

Ap Rees, T. (1966) Aust.J.Biol.Sci. *19*, 981-990.

Ap Rees, T. and Beevers, H. (1960) Plant Physiol. *35*, 830-838.

Atkins, J.F. and Gesteland, R.F. (1975) Molec.Gen.Genet. *139*, 19-31.

Ball, E. (1948) Symp.Soc.Exp.Biol. *2*, 246-262.

Banker, G.A. and Cotman, C.W. (1972) J.Biol.Chem. *247* 5856-5861.

Barlow, P.W. (1974) New Phytol. *73*, 937-954.

Barlow, P.W. (1976) J.Theor.Biol. *57*, 433-451.

Barlow, P.W. (1977) Experientia *33*, 1153-1154.

Barlow, P.W. (1982) in *The Molecular Biology of Plant Development* (Eds. Smith, H. and Grierson, D.) Blackwell, Oxford, pp.185-222.

Barlow, P.W. (1983a) Int.Symp.Gumpenstein 1982, (Ed. W. Bohm *et al.*) pp.711-720.

Barlow, P.W. (1983b) Protoplasma *115*, 1-10.

Barlow, P.W. (1984) In *Positional Controls in Plant Development* (Eds. P.W. Barlow and D.J. Carr), Cambridge University Press.

Barlow, P.W. and MacDonald, P.D.M. (1973) Proc.Roy.Soc.B, *183*, 385-398.

Barlow, P.W., Rost, T.L. and Gunning, B.E.S. (1982) Protoplasma *112*, 205-216.

Barlow, P.W. and Sargent, J.A. (1978) Ann.Bot. *42*, 791-799.

Basha, S.M.M. (1979) Plant Physiol. *63*, 301-306.

Baumann, G. and Chrambach, A. (1975) in *Recent Progress in Isoelectric Focusing and Isotachophoresis* (Ed. P.G. Righetti), Elsevier, Amsterdam.

Beale, E.G., Dedman, J.R. and Means, A.R. (1977) Endocrinology *101*, 1621-1634.

Belitser, N.V., Zaalishvilli, G.V. and Sytnianskaja, N.P. (1982) Protoplasma *111*, 63-78.

Bell, J.K. and McCully, M.E. (1970) Protoplasma *70*, 179-205.

Benoist, C. and Chambon, P. (1981) Nature *290*, 304-310.

Benveniste, K., Wilczek, J., Ruggieri, A. and Stern, R. (1976) Biochem. *15*, 830-835.

Berns, A.J.M., van Kraaikamp, M., Bloemendal, H. and Lane, C.D. (1972) Proc.Nat.Acad.Sci. *69*, 1606-1609.

Berridge, M.J. (1976) in *Calcium in Biological Systems* (Ed. C.J. Duncan) No.XXX Cambridge University Press, New York and London, pp.219-231.

Beyenbach, J., Weber, C. and Kleinig, H. (1974) Planta *119*, 113-124.

Bhakdi, *et al.*(1975) in *Recent Progress in Isoelectric Focussing and Isotachophoresis* (Ed. P.G. Righetti), Elsevier, North Holland, pp.

Biro, R.L., Hale II C.C., Wiegand, O.F. and Roux, S.J. (1982) Ann.Bot. *50*, 737-745.

Bishop, J.O. and Rosbash, M. (1973) Nature New Biol. *241*, 204-207.

Bobb, D. (1973) Annals New York Acad.Sci. *209*, 225-236.

Bonner, W.M. and Laskey, R.A. (1974) Eur.J.Biochem. *46* , 83-88.

Bottomley, W., Higgins, T.J.V. and Whitfield, P.R. (1976) FEBS Letts. *63*, 120-124.

Bouck, G.B. and Cronshaw, J. (1965) J.Cell Biol. *25*, 79-95.

Bowen, M.R., Wilkins, M.B., Lane, A.R. and McCorquodale, I. (1972) Planta *105*, 273-292.

Bradford, M.M. (1976) Anal.Biochem. *72*, 248-254.

Brawerman, G., Mendecki, J. and Lee, S.Y. (1972) Biochem. *11*, 637-646.

Broglie, R., Bellemare, G., Bartlett, S.G., Chua, N.-H. and Cashmore, A.R. (1981) Proc.Nat.Acad.Sci.U.S.A. *78*, 7304-7308.

Brooker, J.D., Tomaszewski, M. and Marcus, A. (1978) Plant Physiol. *61*, 145-149.

Brostrom, C.O. and Wolff, D.J. (1981) Biochem.Pharmacol. *30*, 1395-1405.

Brown, R. (1963) Symp.Soc.Exp.Biol. *17*, 1-17.

Brown, R. (1964) Brookhaven Symposia in Biology *16*, 157-169.

Brown R. and Broadbent, D. (1950) J.Exp.Bot. *1*, 249-263.

Brown, R., Reith, W.S. and Robinson, E. (1952) Symp.Soc.Exp.Biol. *6*, 329-347.

Brown, R. and Rickless, P. (1949) Proc.Roy.Soc.Series B. *136*, 110-125.

Brown, R. and Robinson, E. (1954) J.Exp.Bot. *5*, 71-78.

Brown, R. and Robinson, E. (1955)In *Biological Specificity and Growth* (Ed. Butler, E.G.) Princeton, New Jersey, pp.93-118.

Brown, R. and Sutcliffe, J.F. (1950) J.Exp.Bot. *1*, 88-113.

Bucknall, R.A. and Sutcliffe, J.F. (1965) J.Exp.Bot. *16*, 587-603.

Bull, H.B., Breese, K., Ferguson, G.L. and Swenson, C.A. (1964) Arch.Biochem.Biophys. *104*, 297-304.

Bünning, E. (1952) Survey Biol.Progr. *2*, 105-140.

Burgess, W.H., Howlett, A.C., Kretsinger, R.H. and Gilman, A.G. (1978) J.Cyclic Nuc.Res. *4*, 175-181.

Burgess, W.H., Jemiolo, D.K. and Kretsinger, R.H. (1980) Biochim.Biophys.Acta *623*, 257-270.

Burke, B.E. and DeLorenzo, R.J. (1982) Brain Res. *236*, 393-415.

Burnette, W.N. (1981) Anal.Biochem. *112*, 195-203.

Burström, H.G. (1968) Biol.Rev. *43*, 287-316.

Burström, H.G. (1971) Physiol.Plant. *25*, 509-513.

Burström, H.G. and Svensson, S.B. (1974) In *Structure and Function of Primary Root Tissues* (Ed. J. Kolek), Veda, Bratislava, pp.121-135.

Buvat, R. (1968) C.R.Acad.Sci.(Paris) *267*, 406

Caers, L.I., Peumans, W.J. and Carlier, A.R. (1979) Planta *144*, 491-496.

Caldwell, C.R. and Haug, A. (1980) Physiol.Plant. *50*, 183-193.

Caldwell, C.R. and Haug, A. (1981a) Anal.Biochem. *116*, 325-330.

Caldwell, C.R. and Haug, A. (1981b) Plant Physiol. *67*, suppl.136.

Callow, M.E., Evans, L.V., and Callow, J.A. (1982) in *The Molecular Biology of Plant Development* (Eds. H. Smith and D. Grierson), Blackwell, Oxford, pp.159-181.

Campbell, K.P., MacLennan, D.H. and Jorgensen, A.O. (1983) J.Biol.Chem. *258*, 11267-11273.

Campbell, P.N. and Blobel, G. (1976) FEBS Letts. *72*, 215-226.

Carlin, R.K., Grab, D.J. and Siekevitz, P. (1980) Ann. New York Acad.Sci. *356*, 73-74.

Carrington, C.M.S. and Firn, R.D. (1983) Planta *157*, 524-530.

Cashmore, A.R., Broadhurst, M.K. and Gray, R.E. (1978) Proc. Nat.Acad.Sci. U.S.A. *75*, 655-659.

Catsimpoolas, N. (1973) Ann. New York Acad.Sci. *209*, 65-79.

Catsimpoolas, N. (ed.) (1976) *Isoelectric Focusing*, Academic Press, New York, pp.229-258.

Cereghini, S., Geoghegan, T., Bergmann, I. and Brawerman, G. (1979) Biochem. *18*, 3153-3159.

Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd III, A.E. and Means, A.R. (1982) Cell *28*, 41-50.

Chafouleas, J.G., Dedman, J.R., Munjaal, R.P. and Means, A.R. (1979) J.Biol.Chem. *254*, 10262-10267.

Chaly, N.M. and Setterfield, G. (1975) Can.J.Bot. *53*, 200-218.

Chandler, D.E. and Williams, J.A. (1978a) J.Cell Biol. *76*, 371-385.

Chandler, D.E. and Williams, J.A. (1978b) J.Cell Biol. *76*, 386-399.

Chandra, S., Chabot, J.F., Morrison, G.H. and Leopold, A.C. (1982) Sci. *216*,1221-1223.

Charbonneau, H. and Cormier, M.J. (1979) Biochem.Biophys.Res. Commun. *90*, 1039-1047.

Charbonneau, H., Jarrett, H.W., McCann, R.O. and Cormier, M.J. (1980) In *Calcium Binding Proteins: Structure and Function* (Ed. F.L. Siegel *et al.*), Elsevier, North Holland, pp.155-164.

Charvat, I. and Esau, K. (1975) J.Cell Sci. *19*, 543-561.

Cheung, W.Y. (1967) Biochem.Biophys.Res.Commun. *29*, 478-482.

Cheung, W.Y. (1971) J.Biol.Chem. *246*, 2859-2869.

Cheung, W.Y. (1980) Sci. *207*, 19-27.

Chrambach, A. and Baumann, G. (1976) In *Isoelectric Focusing* (Ed. N. Catsimpoolas), Academic Press, New York, pp.77-91.

Chua, N.-H. and Schmidt, G.W. (1978) Proc.Nat.Acad.Sci.U.S.A. *75*, 6110-6114.

Cimino, M., Prozialeck, W. and Weiss, B. (1979) Pharmacol. *21*, 240.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J.Biol.Chem. *252*, 1102-1106.

Clothier, G. and Timourian, H. (1972) Exp.Cell Res. *75*, 105-110.

Clowes, F.A.L. (1954) New Phytol. *53*, 108-116.

Clowes, F.A.L. (1959) Biol.Rev. *34*, 501-529.

Clowes, F.A.L. (1961) *Apical Meristems*, Blackwell, Oxford.

Coartney, J.S. and Morré, D.J. (1980) Bot.Gaz. *141*, 56-62.

Cohen, J.D. and Nadler, K.D. (1976) Plant Physiol. *57*, 347-350.

Cohen, L.H., Penner, P.E. and Loeb, L.A. (1973) Ann.New York Acad.Sci. *209*, 354-362.

Cohen, P., Klee, C.B., Picton, C. and Shenolikar, S. (1980) Ann.New York Acad.Sci. *356*, 151-161.

Cook, G.M.W. and Stoddart, R.W. (1973) *Surface Carbohydrates of the Eukaryotic Cell*, Academic Press, London.

Cormier, M.J., Anderson, J.M., Charbonneau, H., Jones, H.P. and McCann, R.O. (1980) In *Calcium and Cell Function*, Vol.I (Ed. W.Y. Cheung), Academic Press, New York. pp.201-218.

Cormier, M.J., Charbonneau, H. and Jarrett, H.W. (1981) Cell Calcium *2*, 313-331.

Cormier, M.J., Jarrett, H.W. and Charbonneau, H. (1982) In *Calmodulin and Intracellular Calcium Receptors*, (Eds. S.Kakiuchi, H. Hidaka, and A.R. Means) Plenum Press, New York, pp.125-139.

Cronquist, A.J. (1961) *Introductory Botany*, Harper and Row, New York.

Cronshaw, J. (1965) Can.J.Bot. *43*, 1401-1408.

Cronshaw, J. and Bouck, G.B. (1965) J.Cell Biol. *24*, 415-431.

Cronshaw, J. and Wardrop, A.B. (1964) Aust.J.Bot. *12*, 15-23.

Cumming, A.C. and Lane, B.G. (1979) Eur.J.Biochem. *99*, 217-224.

Cutter, E.G. (1978) *Plant Anatomy*, 2nd Edition, Part I, Arnold, London.

Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochem. *17*, 253-258.

Davidson, E.H. (1976) *Gene Activity in Early Development*, Academic Press, New York.

Davies, D.D. (1980) Adv. in Bot.Res. *8*, 65-126.

Dedman, J.R., Jackson, R.L., Schreiber, W.E. and Means, A.R. (1978) J.Biol.Chem. *253*, 343-346.

Dedman, J.R., Lin, T., Marcum, J.M., Brinkley, B.R. and Means, A.R. (1980) In *Calcium-binding Proteins: Structure and Function*, (Eds. F.L. Siegel *et al.*), Elsevier, North Holland, pp.181-188.

Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D. and Means, A.R. (1977a) J.Biol.Chem. *252*, 8415-8422.

Dedman, J.R., Potter, J.D. and Means, A.R. (1977b) J.Biol.Chem. *252*, 2437-2440.

Dedman, J.R., Welsh, M.J., Kaetzel, M.A., Pardue, R.L. and Brinkley, B.R. (1982) In *Calcium and Cell Function*, Vol.III (Ed. W.Y. Cheung), Academic Press, New York, pp.455-472.

Dedman, J.R., Welsh, M.J. and Means, A.R. (1978) J.Biol.Chem. *253*, 7515-7521.

De la Fuente, R.K. and Leopold, A.C. (1973) Plant Physiol. *51*, 845-847.

Delseny, M., Aspart, L. and Guitton, Y. (1977) Planta *135*, 125-128.

Dennis, D.T. and Miernyk, J.A. (1982) Ann.Rev.Plant Physiol. *33*, 27-50.

Dieter, P. and Marmé, D. (1980a) Ann. New York, Acad.Sci. *356*, 371-373.

Dieter, P. and Marmé, D. (1980b) Cell Calcium *1*, 279-286.

Dieter, P. and Marmé, D. (1980c) In *Calcium-binding Proteins: Structure and Function*, (Eds. F.L. Siegel *et al.*), Elsevier, North Holland, pp.211-212.

Dieter, P. and Marmé, D. (1980d) Proc.Nat.Acad.Sci.U.S.A. *77*, 7311-7314.

Dieter, P. and Marmé, D. (1981a) Biochem.Biophys.Res.Commun. *101*, 749-755.

Dieter, P. and Marmé, D. (1981b) FEBS Letts. *125*, 245-248.

Dieter, P. and Marmé, D. (1983) Planta *159*, 277-281.

Dieter, P. and Marmé, D. (1984) J.Biol.Chem. *259*, 184-189.

Digby, J. and Firn, R.D. (1979) Plant Cell Environ. *2*, 145-148.

Digby, J. and Wareing, P.F. (1966) Ann.Bot. *30*, 539-548.

Dodds, J.H. (1981) Plant, Cell and Env. *4*, 145-146.

Donachie, W.D. (1968) Nature *219*, 1077-1079.

Doohan, M.E. and Palevitz, B.A. (1980) Planta *149*, 389-401.

Dorée, M. and Picard, A. (1980) Experientia *36*, 1291-1292.

Dormer, K.J. (1972) *Shoot Organisation in Vascular Plants* Syracuse University Press, Syracuse, New York.

Drabikowski, W., Kuźnicki, J. and Grabarek, Z. (1978) Comp. Biochem.Physiol. *60*C, 1-6.

Dreyer, E.M. and Weisenseel, M.H. (1979) Planta *146*, 31-39.

Dure III, L., Chlan, C. and Galau, G.A. (1983) In *Structure and Function of the Plant Genome* (Eds. O. Ciferri and L. Dure III), pp.113-121.

Edelstein, C., Lim, C.T. and Scanu, A.M. (1972) J.Biol.Chem. *247*, 5842-5849.

Elias, E. and Boyer, J.L. (1979) Sci. *206*, 1404-1406.

Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. and Hidaka, H. (1981) J.Biol.Chem. *256*, 12485-12489.

Erickson, R.O. and Sax, K.B. (1956a) Proc.Am.Phil.Soc. *100*, 487-498.

Erickson, R.O. and Sax, K.B. (1956b) Proc.Am.Phil.Soc. *100*, 499-514.

Esau, K. (1941) Hilgardia *13*, 437-490.

Esau, K. (1954) Biol.Rev. *29*, 46-87.

Esau, K. (1965a) *Vascular Differentiation in Plants* Holt, Rinehart and Winston, New York.

Esau, K. (1965b) *Plant Anatomy* 2nd Edition, Wiley, New York.

Esau, K. (1974) Ann.Bot. *38*, 159-164.

Esau, K. and Charvat, I. (1978) Ann.Bot. *42*, 665-677.

Esau, K. and Gill, R.H. (1972) J.Ultrastr.Res. *41*, 160-175.

Esnouf, M.P., Klevit, R.E. and Williams, R.J.P. (1980) Ann.New York Acad.Sci. *356*, 380-381.

Etherton, B. (1970) Plant Physiol. *45*, 527-528.

Evans, M.I., Croy, R.R.D., Hutchinson, P., Boulter, D., Payne, P.I. and Gordon, M.E. (1979) Planta *144*, 455-462.

Fazekas de St. Groth, S., Webster, R.G. and Datyner, A. (1963) Biochim.Biophys.Acta *71*, 377-391.

Feldman, L.J. and Torrey, J.G. (1975) Can.J.Bot. *53*, 2796-2803.

Feldman, L.J. and Torrey, J.G. (1976) Am.J.Bot. *63*, 345-355.

Fineran, B.A. (1966) Phytomorphol. *16*, 1-16.

Fineran, B.A. (1970a) J.Ultrastr.Res. *33*, 574-586.

Fineran, B.A. (1970b) Protoplasma *70*, 457-478.

Fineran, B.A. (1971) Protoplasma *72*, 1-18.

Fisher, U. and Albersheim, P. (1973) Plant Physiol. *51*, suppl. 2.

Fiske, C.H. and Subbarow, Y. (1925) J.Biol.Chem. *66*, 375-400.

Fiskum, G. and Lehninger, A.L. (1982) In *Calcium and Cell Function* Vol.III, (Ed. W.Y. Cheung), Academic Press, New York, pp.39-80.

Florini, J.R., Brivio, R.P. and Battelle, B.-A.M. (1973) Ann.New York Acad.Sci. *209*, 299-310.

Foard, D.E. (1959) Nature *184*, 1663-1664.

Fosket, D.E. and Torrey, J.G. (1969) Plant Physiol. *44*, 871-880.

Fowler, M.W. and Ap Rees, T. (1970) Biochim.Biophys.Acta *201*, 33-44.

Francis, D. and MacLeod, R.D. (1977) Ann.Bot. *41*, 1149-1162.

Fry, S.C. and Wangermann, E. (1976) New Phytol. *77*, 313-317.

Gardner, R.L. (1981) In *Cellular Controls in Differentiation* (Eds. C.W. Lloyd and D.A. Rees), Acad.Press, London pp.257-278.

Gariépy, J. and Hodges, R.S. (1983) Biochem. *22*, 1586-1594.

Gerding, J.J.T., Koppers, A., Hagel, P. and Bloemendal, H. (1971) Biochim.Biophys.Acta *243*, 374-379.

Gibbs, M. and Beevers, H. (1955) Plant Physiol. *30*, 343-347.

Gibbs, M. and Earl, J.M. (1959) Plant Physiol. *34*, 529-532.

Gilkey, J.C., Jaffe, L.F., Ridgway, E.B. and Reynolds, G.T. (1978) J.Cell Biol. *76*, 448-466.

Glenney, J.R., Bretscher, A. and Weber, K. (1980) Proc.Nat. Acad.Sci.U.S.A. *77*, 6458-6462.

Goff, C.W. (1975) Am.J.Bot. *62*, 280-291.

Goldsmith, M.H.M. (1977) Ann.Rev.Plant Physiol. *28*, 439-478.

Goodwin, R.H. and Avers, C.J. (1956) Am.J.Bot. *43*, 479-487.

Goodwin, R.H. and Stepka, W. (1945) Am.J.Bot. *32*, 36-46.

Gopalakrishna, R. and Anderson, W.B. (1982) Biochem.Biophys. Res.Commun. *104*, 830-836.

Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem.Biophys. Res.Commun. *77*, 1203-1209.

Goswami, K.A. and Audus, L.J. (1976) Ann.Bot. *40*, 49-64.

Graf, E., Filoteo, A.G. and Penniston, J.T. (1980) Arch.Biochem. Biophys. *203*, 719-726.

Graham, C.F. and Wareing, P.F. (1976) *The Developmental Biology of Plants and Animals* (Eds. C.F. Graham and P.F. Wareing), Blackwell, Oxford, pp.45-48.

Grand, R.J.A., Nairn, A.C. and Perry, S.V. (1980) Biochem.J. *185*,755-760.

Grand, R.J.A. and Perry, S.V. (1978) FEBS Letts. *92*, 137-142.

Grand, R.J.A. and Perry, S.V. (1979) Biochem.J. *183*, 285-295.

Grand, R.J.A., Perry, S.V. and Weeks, R.A. (1979) Biochem.J. *177*, 521-529.

Greenwood, M.S., Hillman, J.R., Shaw, S. and Wilkins, M.B. (1973) Planta *109*, 369-374.

Grefrath, S.P. and Reynolds, J.A. (1974) Proc.Nat.Acad.Sci. U.S.A. *71*, 3913-3916.

Griffing, L.R. and Ray, P.M. (1979) Plant Physiol. *63*, suppl. 283.

Grison, R. and Pilet, P.E. (1978) Plant Sci.Letters *13*, 213-218.

Gross, J. and Marmé, D. (1978) Proc.Nat.Acad.Sci.U.S.A. *75*, 1232-1236.

Gruss, P., Dhar, R. and Khoury, G. (1981) Proc.Nat.Acad.Sci. U.S.A. *78*, 943-947.

Gunning, B.E.S. and Hardham, A.R. (1982) Ann.Rev.Plant Physiol. *33*, 651-698.

Hagel, P., Gerding, J.J.T., Fieggen, W. and Bloemendal, H. (1971) Biochim.Biophys.Acta *243*, 366-373.

Hagemann, R. (1956) Die Kulturpflanze *4*, 46-82.

Hahlbrock, K. and Grisebach, H. (1979) Ann.Rev.Plant Physiol. *30*, 105-130.

Haiech, J., Derancourt, J., Pechère, J.-F. and Demaille, J.G. (1979) Biochem. *18*, 2752-2758.

Haines, M.E., Carey, N.H. and Palmiter, R.D. (1974) Eur.J. Biochem. *43*, 549-560.

Halbreich, A. (1979) Biochem.Biophys.Res.Commun. *86*, 78-87.

Hale II, C.C. and Roux, S.J. (1980) Plant Physiol. *65*, 658-662.

Hall, J.L., Flowers, T.J. and Roberts, R.M. (1974) *Plant Cell Structure and Metabolism*. Longman, London.

Hall, J.L. and Sexton, R. (1972) Planta *108*, 103-120.

Hammett, J.R. and Katterman, F.R. (1975) Biochem. *14*, 4375-4379.

Hanstein, J. (1868) Festschr.Niederrhein.Gesellschaft Nat. Heilk.Zum 50-Jahr Jubilaüm Univ. Bonn 109-143.

Hardham, A.R. (1982) in *The Cytoskeleton in Plant Growth and Development* (ed. C.W. Lloyd) Academic Press, London pp.377-403.

Hardham, A.R. and McCully, M.E. (1982) Protoplasma *112*, 152-166.

Harris, P. (1975) Exp.Cell Res. *94*, 409-425.

Harris, P.J. and Northcote, D.H. (1970) Biochem.J. *120*, 479-491.

Harris, S.E., Rosen, J.M., Means, A.R. and O'Malley, B.W. (1975) Biochem. *14*, 2072-2081.

Hawes, C.R., Juniper, B.E. and Horne, J.C. (1981) Planta *152*, 397-407.

Head, J.F., Weeks, R.A. and Perry, S.V. (1977) Biochem.J. *161*, 465-471.

Heber, U.W. and Santarius, K.A. (1965) Biochim.Biophys.Acta *109*, 390-408.

Heimsch, C. (1951) Am.J.Bot. *38*, 523-537.

Hejnowicz, Z. (1959) Physiol.Plant *12*, 124-138.

Helenius, A. and Simons, K. (1975) Biochim.Biophys.Acta *415*, 29-79.

Hepler, P.K. (1980) J.Cell Biol. *86*, 490-499.

Hepler, P.K. (1982) Protoplasma *111*, 121-133.

Hepler, P.K. and Palevitz, B.A. (1974) Ann.Rev.Plant Physiol. *25*, 309-362.

Herman, I.M. and Pollard, T.D. (1979) J.Cell Biol. *80*, 509-520.

Herson, D., Schmidt, A., Seal, S., Marcus, A. and van Vloten-Doting, L. (1979) J.Biol.Chem. *254*, 8245-8249.

Herth, W. (1978) Protoplasma *96*, 275-282.

Heslop-Harrison, J. (1967) Ann.Rev.Plant Physiol. *18*, 325-348.

Hetherington, A. and Trewavas, A. (1982) FEBS Letts. *145*, 67-71.

Heyes, J.K. (1960) Proc.Roy.Soc.Lond.Series B, *152*, 218-230.

Heyes, J.K. (1977) In *The Physiology of the Garden Pea* (eds. J.F. Sutcliffe and J.S. Pate) Academic Press, London, pp.153-181.

Heyes, J.K. and Brown, R. (1965) Enc.Plant Physiol. *15*(1), 189-212.

Heyser, W. (1971) Cytobiologie *4*, 186-197.

Higginbotham, N. (1973) Ann.Rev.Plant Physiol. *24*, 25-46.

Higgins, T.J.V., Zwar, J.A. and Jacobsen, J.V. (1976) Nature *260*, 166-168.

Higuichi, T. and Shimada, M. (1967a) Plant Cell Physiol. *8*, 61-69.

Higuichi, T. and Shimada, M. (1967b) Plant Cell Physiol. *8*, 71-78.

Hipkins, M.F. (1983) In *Metals and Micronutrients; Uptake and Utilisation by Plants* (eds. D.A. Robb and W.S. Pierpoint), Academic Press, pp.147-168.

Hodges, T.K. and Hanson, J.B. (1965) Plant Physiol. *40*, 101-109.

Hooper, J.E. and Kelly, R.B. (1984) J.Biol.Chem. *259*, 148-153.

Horecker, B.L. (1962) Harvey Lectures Series *57*, Academic Press, New York, pp.35-61.

Howe, J.G. and Hershey, J.W.B. (1981) J.Biol.Chem. *256*, 12836-12839.

Hunter, A.R., Farrell, P.J., Jackson, R.J. and Hunt, T. (1977) Eur.J.Biochem. *75*, 149-157.

Hutchinson, T.E., Cantino, M.E. and Cantino, E.C. (1977) Biochem.Biophys.Res.Commun. *74*, 336-342.

Hyams, J.S. (1982) In *The Cytoskeleton in Plant Growth and Development* (ed. C.W. Lloyd), Academic Press, London pp.31-53.

Igarashi, K., Watanabe, Y. and Hirose, S. (1975) Biochem. Biophys.Res.Commun. *67*, 407-413.

Igarashi, K., Watanabe, Y., Nakamura, K., Kojima, M., Fujiki, Y. and Hirose, S. (1978) Biochem.Biophys.Res.Commun. *83*, 806-813.

Igarashi, K., Yabuki, M., Yoshioka, Y., Eguchi, K. and Hirose, S. (1977) Biochem.Biophys.Res.Commun. *75*, 163-171.

Jacob, F. and Monod, J. (1961) J.Mol.Biol. *3*, 318-356.

Jacobs, S. (1973) Analyst *98*, 25-33.

Jacobs, W.P. (1952) Am.J.Bot. *39*, 301-309.

Jacobs, W.P. (1954) Am.Naturalist *88*, 327-337.

Jacobs, W.P. and Morrow, I.B. (1957) Am.J.Bot. *44*, 823-842.

Jaffe, L.F. (1966) Proc.Nat.Acad.Sci.U.S.A. *56*, 1102-1109.

Jaffe, L.F. (1968) Adv.Morphog. *7*, 295-328.

Jaffe, L.F. and Nuccitelli, R. (1977) Ann.Rev.Biophys.Bioeng. *6*, 445-476.

Jaffe, L.F., Robinson, K.R. and Nuccitelli, R. (1974) Ann. New York Acad.Sci. *238*, 372-389.

Jaffe, L.A., Weisenseel, M.H. and Jaffe, L.F. (1975) J.Cell Biol. *67*, 488-492.

Jamieson, G.A., Jr., and Vanaman, T.C. (1979) Biochem.Biophys. Res.Commun. *90*, 1048-1056.

Janczewski, M.E. de (1874) Ann.Sci.Nat.Bot. 5^{e} Ser. *20*, 162-201.

Jarrett, H.W., Brown, C.J., Black, C.C. and Cormier, M.J. (1982a) J.Biol.Chem. *257*, 13795-13804.

Jarrett, H.W. Charbonneau, H., Anderson, J.M., McCann, R.O. and Cormier, M.J. (1980) Ann.New York Acad.Sci. *356*, 119-129.

Jarrett, H.W., DaSilva, T. and Cormier, M.J. (1983) In *Metals and Micronutrients: Uptake and Utilisation of Metals in Plants*. (eds. D.A. Robb and D.S. Pierpoint), Academic Press, London, pp.205-218.

Jarrett, H.W. and Penniston, J.T. (1978) J.Biol.Chem. *253*, 4676-4682.

Jarvis, L.R. and Wardrop, A.B. (1974) Planta *119*, 101-112.

Jeffs, R.A. and Northcote, D.H. (1966) Biochem.J. *101*, 146-152.

Jemiolo, D.K., Burgess, W.H., Rebhun, L.I. and Kretsinger, R.H. (1980) J.Cell Biol. *87*, 248a.

Jenkinson, I.S. (1962a) Aust.J.Biol.Sci. *15*, 101-114.

Jenkinson, I.S. (1962b) Aust.J.Biol.Sci. *15*, 115-125.

Jensen, W.A. (1955a) Plant Physiol. *30*, 426-432.

Jensen, W.A. (1955b) Exp.Cell Res. *8*, 506-522.

Jensen, W.A. (1958) Exp.Cell Res. *14*, 575-583.

Jensen, W.A. and Kavaljian,L.G.(1958) Am.J.Bot. *45*, 365-372.

Job, D., Fischer, E.H. and Margolis, R.L. (1981) Proc.Nat. Acad.Sci. *78*, 4679-4682.

Jones, H.P., Lenz, R.W., Palevitz, B.A. and Cormier, M.J. (1980) Proc.Nat.Acad.Sci.U.S.A. *77*, 2772-2776.

Kafatos, F.C. (1972) Karolinska Symposium on Research Methods in Reproductive Endocrinology. Periodica, Copenhagen, pp.319-345.

Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K., Yasuda, S., Kashiba, A., Sobue, K., Oshima, M. and Nakajima, T. (1978) Adv.Cyc.Nuc.Res. *9*, 253-263.

Kaplan, L.J. and Foster, J.F. (1971) Biochem. *10*, 630-636.

Kern, H. (1975) Anal.Biochem. *67*, 147-156.

Khavkin, E.E., Zelena, I.V. and Varakina, N.N. (1974) In *Structure and Function of Primary Root Tissues.* (ed. J.Kolek), Veda, Bratislava, pp.221-225.

Kiehart, D.P. (1981) J.Cell Biol. *88*, 604-617.

Kiehart, D.P., Inoué, S. and Mabuchi, I. (1977) J.Cell Biol. *75*, 258a.

Kincaid, R.L., Manganiello, V.C. and Vaughan, M. (1979) Fed.Proc. *38*, 788.

Kirby, K.S. (1965) Biochem. J. *96*, 266-269.

Klee, C.B. (1977) Biochem. *16*, 1017-1024.

Klee, C.B. (1980) In *Calcium and Cell Function* Vol.I (ed. W.Y. Cheung), pp.59-77.

Klee, C.B., Crouch, T.H. and Krinks, M.H. (1979a) Proc.Nat. Acad.Sci.U.S.A. *76*, 6270-6273.

Klee, C.B., Crouch, T.H. and Krinks, M.H. (1979b) Biochem. *18*, 722-729.

Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Ann.Rev. Biochem. *49*, 489-515.

Kleinig, H. (1975) Planta *127*, 163-170.

Kloppstech, K. (1982) In *The Molecular Biology of Plant Development* (eds. H.Smith and D. Grierson), Blackwell, Oxford, pp.136-158.

Kovalev, A.G. and Obrucheva, N.V. (1976) Fiziol.Rastenii, *23*, 290-295 (English translation).

Kovalev, A.G. and Obrucheva, N.V. (1977) Ontogenez *8*, 397-405.

Krause, G.H. and Heber, U. (1976) in *The Intact Chloroplast* (ed. J. Barber), Elsevier, North Holland, pp.171-214.

Kretsinger, R.H. (1980) CRC Crit.Rev.Biochem. *8*, 119-174.

Kubowicz, B.D., Vanderhoef, L.N. and Hanson, J.B. (1982) Plant Physiol. *69*, 187-191.

Kumagai, H. and Nishida, E. (1979) J.Biochem. *85*, 1267-1274.

Laemmli, U.K. (1970) Nature *227*, 680-685.

Lambin, P. (1978) Anal.Biochem. *85*, 114-125.

Lambin, P. and Fine, J.M. (1979) Anal.Biochem. *98*, 160-168.

Lambin, P., Rochu, D. and Fine, J.M. (1976) Anal.Biochem. *74*, 567-575.

Larkins, B.A., Jones, R.A. and Tsai, C.Y. (1976) Biochem. *15*, 5506-5511.

Larkins, B.A., Pederson, K., Marks, M.D., Wilson, D.R. and Argos, P. (1983) In *Structure and Function of the Plant Genome*. (eds. O. Ciferri and L. Dure III), pp.73-83.

Laskey, R.A. and Mills, A.D. (1975) Eur.J.Biochem. *56*, 335-341.

Leaver, C.J. and Ingle, J. (1971) Biochem.J. *123*, 235-243.

Leech, J.H., Mollenhauer, H.H. and Whaley, W.G. (1963) Symp.Soc.Exp.Biol. *17*, 74-84.

Leech, R.M. and Murphy, D.J. (1976) In *The Intact Chloroplast* (ed. J. Barber), Elsevier, North Holland, pp.365-401.

Les-Huang, S. and Ochoa, S. (1971) Nature New Biol. *234*, 236-239.

Lendzian, K.J. (1978) Planta *141*, 105-110.

Lendzian, K.J. and Bassham, J.A. (1975) Biochim.Biophys.Acta *396*, 260-275.

Leonard, R.T. (1983) In *Metals and Micronutrients: Uptake and Utilisation by Plants* (eds. D.A. Robb and W.S. Pierpoint) Academic Press, London, pp.71-86.

Leshem, Y.Y., Würzburger, Y. and Frimer, A.A., Barness, G. and Ferguson, I.B. (1982) Eleventh Int.Conf. on Plant Growth Substances 1982 (ed. P.F. Wareing) Academic Press, London.

Levin, D.H., Kyner, D. and Acs, G. (1973) J.Biol.Chem. *248*, 6416-6425.

Levin, R.M. and Weiss, B. (1976) Mol.Pharmacol. *12*, 581-589.

Levin, R.M. and Weiss, B. (1977) Mol.Pharmacol. *13*, 690-697.

Levin, R.M. and Weiss, B. (1978) Biochim.Biophys.Acta *540*, 197-204.

Levin, R.M. and Weiss, B. (1979) J.Pharmacol.Exp.Ther.*208*, 454-459.

Liang, S.-M., Liang, C.-M. and Liu, T.-Y. (1981) J.Biol.Chem. *256*, 4968-4972.

Libbenga, K.R., van Iren, F., Bogers, R.J. and Schraag-Lamers, M.F. (1973) Planta *114*, 29-39.

Lin, C.T., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1980) J.Cell Biol. *85*, 473-480.

Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1974) J.Biol.Chem. *249*, 4943-4954.

Lin, Y.M., Liu, Y.P. and Cheung, W.Y. (1975) FEBS Lett *49*, 356-360.

Link, G., Coen, D.M. and Bogorad, L. (1978) Cell *15*, 725-731.

Lintilhac, P.M. (1974a) Am.J.Bot. *61*, 135-140.

Lintilhac, P.M. (1974b) Am.J.Bot. *61*, 230-237.

Lintilhac, P.M. and Vesecky, T.B. (1980) Am.J.Bot. *67*, 1477-1483.

Lloyd, C.W. and Barlow, P.W. (1982) In *The Cytoskeleton in Plant Growth and Development* (ed. C.W. Lloyd), Academic Press, London, pp.203-228.

Lloyd, C.W. and Rees, D.A. (1981) *Cellular Controls in Differentiation*, Academic Press, London.

Lodish, H.F. (1970) J.Mol.Biol. *50*, 689-702.

Lodish, H.F. (1971) J.Biol.Chem.*246*, 7131-7138.

Lodish, H.F. (1974) Nature *251*, 385-388.

Lodish, H.F. and Jacobsen, M. (1972) J.Biol.Chem. *247*, 3622-3629.

Loehning, U.E. (1961) Biochem.J. *81*, 254-260.

Lomedico, P.T. and Saunders, G.F. (1977) Sci. *198*, 620-622.

Longacre, S.S. and Rutter, W.J. (1977) J.Biol.Chem. *252*, 2742-2752.

Lovett, J.S. (1975) Bacteriol Rev. *39*, 345-404.

Lovett, J.S. (1982) In *The Molecular Biology of Plant Development* (eds. H. Smith and D. Grierson), Blackwell, Oxford, pp.113-135.

Loewenstein, W.R., Kanno, Y. and Ito, S. (1966) Ann. New York Acad.Sci. *137*, 708-716.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. *193*, 265-275.

Luckaser, J.R., White, J.G. and Kersey, J.H. (1974) Proc.Nat. Acad.Sci.U.S.A. *71*, 5088-5090.

Lynch, T.J., Tallant, E.A. and Cheung, W.Y. (1976) Biochem. Biophys.Res.Commun. *68*, 616-625.

Lyndon, R.F. (1977) In *The Physiology of the Garden Pea* (eds. J.F. Sutcliffe and J.S. Pate), Academic Press, London, pp.183-211.

Lyndon, R.F. (1979) In *Differentiation and the Control of Development in Plants-Potential for Chemical Modification* (ed. E.C. George), BPGRG Monograph No.3, Wessex Press, Wantage, pp.57-73.

MacLeod, R.D. (1976) New Phytol. *77*, 747-755.

MacManus, J.P. (1981) FEBS Lett. *126*, 245-249.

Mans, R.J. and Novelli, G.D. (1961) Arch.Biochem.Biophys. *94*, 48-53.

Marcu, K. and Dudock, B. (1974) Nuc.Acids Res. *1*, 1385-1397.

Marcum, J.M., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1978) Proc.Nat.Acad.Sci.U.S.A. *75*, 3771-3775.

Marcus, A., Seal, S.N. and Weeks, D.P. (1974) Methods in Enzymol. *XXX*, 94-101.

Marshak, D.R., Watterson, D.M. and Van Eldik, L.J. (1981) Proc.Nat.Acad.Sci.U.S.A. *78*, 6793-6797.

Matile, P. (1968) Planta *79*, 181-196.

Matile, P. (1975) *The Lytic Compartment of Plant Cells* Wien.

Matile, P. and Moor, H. (1968) Planta *80*, 159-175.

Mazia, D., Petzelt, C., Williams, R.O. and Meza, I. (1972) Exp.Cell Res. *70*, 325-332.

McClurkin, I.T. and McClurkin, D.C. (1967) Plant Physiol. *42*, 1103-1110.

McKeehan, W.L. (1974) J.Biol.Chem. *249*, 6517-6526.

McKnight, G.S., Pennequin, P. and Schimke, R.T. (1975) J.Biol.Chem. *250*, 8105-8110.

Means, A.R. and Chafouleas, J.G. (1983) Cell Biol.Int.Rep. *7*, 481-482.

Means, A.R. and Dedman, J.R. (1980) Nature *285*, 73-77.

Meindl, U. (1982) Protoplasma *112*, 138-141.

Meins, Jr. F. (1975) In *The Cell Cycle and Cell Differentiation* (eds. J. Reinert and H. Holtzer), Springer-Verlag, Berlin, pp.151-175.

Mesquita, J.F. (1969) J.Ultrastr.Res. *26*, 242-250.

Metcalfe, D. (1981) In *Cellular Controls in Differentiation* (eds. C.W. Lloyd and D.A. Rees), Academic Press, London pp.125-144.

Miflin, B.J., Rahman, S., Kreis, M., Forde, B.G., Blanco, C. and Shewry, P.R. (1983) In *Structure and Function of the Plant Genome* (eds. O. Ciferri and L. Dure III) pp.85-92.

Miginiac-Maslow, M. and Hoarau, A. (1977) Plant Sci.Lett. *9*, 7-15.

Miller, J.H. (1980) Am.J.Bot. *67*, 534-542.

Mimura, T. and Tazawa, M. (1983) Protoplasma *118*, 49-55.

Moll, C. and Jones, R.L. (1981) Planta *152*, 450-456.

Monod, J. and Jacob, F. (1961) Cold Spring Harbor Symp.Quant. Biol. *26*, 389-401.

Moore, A.E. and Stone, B.A. (1972) Planta *104*, 93-109.

Moreau, M., Guerrier, P. and Dorée, M. (1978) Nature *272*, 251-252.

Morris, D.A., Kadir, G.O. and Barry, A.J. (1973) Planta *110*, 173-182.

Mulkey, T.J. and Evans, M.L. (1981) Sci. *212*, 70-71.

Muto, S. (1982) FEBS Lett. *147*, 161-164.

Muto, S. (1983) Z. Pflanzenphysiol. *109*, 385-393.

Muto, S., Izawa, S. and Miyachi, S. (1982) FEBS Lett. *139*, 250-254.

Muto, S. and Miyachi, S. (1977) Plant PHysiol. *59*, 55-60.

Muto, S., Miyachi, S., Usada, H., Edwards, G.E. and Bassham, J.A. (1981) Plant Physiol. *68*, 324-328.

Narayanaswamy, S. (1977) In *Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture* (eds. J. Reinert and Y.P.S. Bajaj), Springer-Verlag, Berlin, pp.179-248.

Navarette, M.H. and Bernabeu, C. (1978) Planta *142*, 147-151.

Niemczura, W.P., Tindall, S.H. and Gibbons, W.A. (1982) Fed.Proc. *41*, 502.

Nishida, E., Kumagai, H., Ohtsuki, I. and Sakai, H. (1979) J.Biochem. *85*, 1257-1266.

Nobel, P.S. (1967) Nature *214*, 875-877.

Nordheim, A. and Rich, A. (1983) Nature *303*, 674-679.

Northcote, D.H. (1979) In *Differentiation and the Control of Development in Plants-Potential for Chemical Modification* (ed. E.C. George), BPGRG Monograph No.3, Wessex Press, Wantage, pp.11-20.

Northcote, D.H. and Pickett-Heaps, J.D. (1966) Biochem.J. *98*, 159-167.

Nuss, D.L. and Koch, G. (1976) J.Mol.Biol. *102*, 601-612.

Oaks, A. (1965a) Plant Physiol. *40*, 142-149.

Oaks, A. (1965b) Plant Physiol. *40*, 149-155.

O'Brien, T.P. (1982) In *The Molecular Biology of Plant Development* (eds. H. Smith and D. Grierson) Blackwell, Oxford, pp.49-109.

O'Brien, T.P., Kuo, J., McCully, M.E. and Zee, S.-Y. (1973) Aust.J.Biol.Sci. *26*, 1231-1250.

O'Farrell, P.H. (1975) J.Biol.Chem. *250*, 4007-4021.

O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell *12*, 1133-1141.

Ogren, W.L. and Krogmann, D.W. (1965) J.Biol.Chem. *240*, 4603-4608.

Oh-Hama, T., Miyachi, S. and Tamiya, H. (1963) In *La Photosynthese* CNRS, Paris, 439-448.

Osmond, C.B. and Ap Rees, T. (1969) Biochim.Biophys.Acta *184*, 35-42.

Palmiter, R.D. (1973) J.Biol.Chem. *248*, 8260-8270.

Palmiter, R.D. (1974) Biochem. *13*, 3606-3615.

Palmiter, R.D. (1975) Cell *4*, 189-197.

Park, C.M. (1973) Ann.New York Acad.Sci. *209*, 237-257.

Pardue, R.L., Kaetzel, M.A., Hahn, S.H., Brinkley, B.R. and Dedman, J.R. (1981) Cell *23*, 533-542.

Peterson, B.M., Roberts, B.E. and Yaffe, D. (1974) Proc.Nat. Acad.Sci.U.S.A. *71*,4467-4471.

Paul, D.C. and Goff, C.W. (1973) Exp.Cell Res. *78*, 399-413.
Payvar, F. and Schimke, R.T. (1979) J.Biol.Chem. *254*, 7636-7642.
Perrie, W.T. and Perry, S.V. (1970) Biochem.J. *119*, 31-38.
Perrie, W.T., Smillie, L.B. and Perry, S.V. (1973) Biochem.J. *135*, 151-164.
Peterson, R.L. (1967) Can.J.Bot. *45*, 319-331.
Petzelt, C. (1972) Exp.Cell Res. *70*, 333-339.
Peumans, W.J. and Carlier, A.R. (1977) Planta *136*, 195-201.
Pichard, A.-L. and Cheung, W.Y. (1977) J.Biol.Chem. *252*, 4872-4875.
Pilet, P.-E., Elliot, M.C. and Molony, M.M. (1979) Planta *146*, 405-408.
Pitt-Rivers, R. and Impiombato, F.S.A. (1968) Biochem.J. *109*, 825-830.
Popham, R.A. (1955) Am.J.Bot. *42*, 529-540.
Poux, N. (1962) J.Microscopie *1*, 55-66.
Prozialeck, W.C., Cimino, M. and Weiss, B. (1981) Mol. Pharmacol. *19*, 264-269.
Prozialeck, W.C. and Weiss, B. (1982a) J.Pharmacol Exp. Ther. *222*, 509-516.
Prozialeck, W.C. and Weiss, B. (1982b) Fed.Proc. *41*, 1566.
Pryke, J.A. and Ap Rees, T. (1976) Planta *132*, 279-284.
Quader, H. and Robinson, D.G. (1979) Eur.J.Cell Biol. *20*, 51-56.
Quail, P.H. (1979) Ann.Rev.Plant Physiol. *30*, 425-484.
Raff, E.C., Fuller, M.T., Kaufman,T.C., Kempheus, K.J., Rudolf, J.E. and Raff, R.A. (1982) Cell *28*, 33-40.
Rayle, D.L. and Cleland, R. (1977) Curr.Topics Devel.Biol. *11*, 187-214.
Reinhard, E. (1953) Z.Bot. *42*, 353-376.
Reiss, H.-D. and Herth, W. (1979a) Planta *145*, 225-232.
Reiss, H.-D. and Herth, W. (1979b) Planta *146*, 615-621.
Reynolds, E.S. (1963) J.Cell Biol. *17*, 208-213.
Righetti, P.G. (ed.) (1975) *Recent Progress in Isoelectric Focusing and Isotachophoresis* Elsevier, Amsterdam.
Rink, T.J., Smith, S.W. and Tsien, R.Y. (1982) FEBS Lett. *148*, 21-26.

Rivier, C. and Pilet, P.-E. (1974) Planta *120*, 107-112.

Roberts, B.E. and Paterson, B.M. (1973) Proc.Nat.Acad.Sci. U.S.A. *70*, 2330-2334.

Robinson, A.B. and Rudd, C.J. (1974) Curr.Top.Cell Regul. *8*, 247-295.

Robinson, D.G. and Quader, H. (1982) In *The Cytoskeleton in Plant Growth and Development* (ed. C.W. Lloyd) Academic Press, London, pp.109-126.

Robinson, E. and Brown, R. (1952) J.Exp.Bot. *3*, 356-374.

Robinson, K.R. and Jaffe, L.F. (1975) Sci. *187*, 70-72.

Rosenbusch, J.P. (1974) J.Biol.Chem. *249*, 8019-8029.

Ross, J., Gielen, J., Packman, S., Ikawa, Y. and Leder, P. (1974) J.Mol.Biol. *87*, 697-714.

Roux, S.J. and Slocum, R.D. (1982) In *Calcium and Cell Function* Vol.III (ed. W.Y. Cheung), Academic Press, New York, pp.409-453.

Sachs, J. (1873) Arb.Bot.Inst. Würzburg *1*, 385-474.

Sachs, T. (1968) Ann.Bot. *32*, 781-790.

Sachs, T. (1969) Ann.Bot. *33*, 263-275.

Sachs, T. (1975) Ann.Bot. *39*, 197-204.

Sachs, T. (1975) Planta *127*, 201-206.

Sachs, T. (1978) Differentiation *11*, 65-73.

Sahulka, J. (1974) In *Structure and Function of Primary Root Tissues* (ed. J. Kolek), Veda, Bratislava, pp.235-237.

Saitoh, Y., Wells, J.N. and Hardham, J.G. (1982) Fed.Proc. *41*, 1728.

Salimath, B.P. and Marmé, D. (1983) Planta *158*, 560-568.

Sasaki, Y., Ishiye, M., Sakihama, T. and Kamikubo, T. (1981) J.Biol.Chem. *256*, 2315-2320.

Saunders, M.J. and Hepler, P.K. (1981) Planta *152*, 272-281.

Savíc, A. and Poccia, D. (1978) Anal.Biochem. *88*, 573-579.

Scanu, A.M., Edelstein, C. and Aggerbeck, L. (1973) Ann. New York Acad.Sci *209*, 311-327.

Scharff, O. (1981) Cell Calcium *2*, 1-27.

Schleicher, M., Zendegui, J.G. and Watterson, D.M. (1982a) Fed.Proc. *41*, 503.

Schleicher, M. Iverson, D.B., Van Eldik, L.J. and Watterson, D.M. (1982b) In *The Cytoskeleton in Plant Growth and Development* (ed. C.W. Lloyd), Academic Press, London pp.85-106.

Schnarrenberger, C., Tetour, M. and Herbert, M. (1975) Plant Physiol. *56*, 836-840.

Schreier, M.H. and Staehelin, T. (1973) Proc.Nat.Acad.Sci. U.S.A. *70*, 462-465.

Schröder, J., Kreuzaler, F., Schäfer, E. and Hahlbrock, K. (1979) J.Biol.Chem. *254*, 57-65.

Scott, B.I.H. and Martin, D.W. (1962) Aust.J.Biol.Sci. *15*, 83-100.

Scott Russell, R. (1977) *Plant Root Systems* McGraw-Hill, United Kingdom.

Seago, J.L. (1973) Am.J.Bot. *60*, 607-618.

Seamon, K.B. (1980) Biochem. *19*, 207-215.

Sexton, R. and Sutcliffe, J.F. (1969) Ann.Bot. *33*, 683-694.

Shafritz, D.A. (1977) In *Molecular Mechanisms of Protein Biosynthesis* (eds. H. Weissbach and S. Pestka), Academic Press, New York, pp.555-601.

Shapiro, A.L., Viñuela, E. and Maizel, J.V. (1967) Biochem. Biophys.Res.Commun. *28*, 815-820.

Shapiro, D.J. and Baker, H.J. (1977) J.Biol.Chem. *252*, 5244-5250.

Shapiro, D.J., Baker, H.J. and Stitt, D.T. (1976) J.Biol.Chem. *251*, 3105-3111.

Sharma, R.K., Wirch, E. and Wang, J.H. (1978a) J.Biol.Chem. *253*, 3575-3580.

Sharma, R.K., Desai, R., Thompson, T.R. and Wang, J.H. (1978b) Can.J.Biochem. *56*, 598-604.

Sharma, R.K., Desai, R., Waisman, D.M. and Wang, J.H. (1979) J.Biol.Chem. *254*, 4276-4282.

Sheldrake, A.R. (1973) Biol.Rev. *48*, 509-559.

Silver, R.B., Cole, R.D. and Cande, W.Z. (1980) Cell *19*, 505-516.

Slocum, R.D. and Roux, S.J. (1982) J.Hist.Cytochem. *30*, 617-629.

Slocum, R.D. and Roux, S.J. (1983) Planta *157*, 481-492.

Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Lett. *99*, 199-202.

Sobue, K., Fujita, M., Muramoto, Y. and Kakiuchi, S. (1981a) FEBS Lett. *132*, 137-140.

Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981b) Proc.Nat.Acad.Sci. *78*, 5652-5655.

Soliday, C.L. and Kolattukudy, P.E. (1977) Plant Phys. *59*, 1116-1121.

Soll, D.R. and Sonneborn, D.R. (1969) Devel.Biol. *20*, 218-235.

Soll, D.R. and Sonneborn, D.R. (1972) J.Cell Sci. *10*, 315-333.

Sonenshein, G.E. and Brawerman, G. (1976) Biochem. *15*, 5501-5506.

Sonenshein, G.E. and Brawerman, G. (1977) Biochem. *16*, 5445-5448.

Spiegel, S. and Marcus, A. (1975) Nature *256*, 228-230.

Stange, L. (1965) Ann.Rev.Plant Physiol. *16*, 119-140.

Stark, G.R., Stein, W.H. and Moore, S. (1960) J.Biol.Chem. *235*, 3177-3181.

Steeves, T.A. and Sussex, I.M. (1972) *Patterns in Plant Development*, Prentice-Hall, Englewood Cliffs, New Jersey.

Steffensen, D.M. (1977) In Chromosomes Today *6*, (ed. A. de la Chapelle and M. Sorsa), Elsevier, North Holland, pp.247-253.

Steinhardt, R.A. and Epel, D. (1974) Proc.Nat.Acad.Sci. U.S.A. *71*, 1915-1919.

Stevens, F.C., Walsh, M., Ho, H.C., Teo, T.S. and Wang, J.H. (1976) J.Biol.Chem. *251*, 4495-4500.

Stevenson, T.T. and Cleland, R.E. (1981) Plant Physiol. *67*, 749-753.

Steward, F.C., Ammirato, P.V. and Mapes, M.O. (1970) Ann.Bot. *34*, 761-787.

Steward, F.C., Lyndon, R.F. and Barber, J.T. (1965) Am.J.Bot. *52*, 155-164.

Sundberg, L. and Porath, J. (1974) J.Chromatography *90*, 87-98.

Sunderland, N. and McLeish, J. (1961) Exp.Cell Res. *24*, 541-554.

Sutcliffe, J.F. and Sexton, R. (1969) In *Root Growth* (ed. W.J. Whittington), Butterworths, London, pp.80-102.

Sutcliffe, J.F. and Sexton, R.F. (1974) In *Structure and Function of Primary Root Tissues*, (ed. J. Kolek), Veda, Bratislava, pp.203-219.

Suzuki, Y. and Suzuki, E. (1974) J.Mol.Biol. *88*, 393-407.

Svensson, H. (1961) Acta Chem.Scand. *15*, 325-341.

Switzer, R.C., Merril, C.R. and Shifrin, S. (1979) Anal. Biochem. *98*, 231-237.

Tanaka, T. and Hidaka, H. (1980) J.Biol.Chem. *255*, 11078-11080.

Taylor, J.M. (1979) Ann.Rev.Biochem. *48*, 681-717.

Teo, T.S., Wang, T.H. and Wang, J.H. (1973) J.Biol.Chem. *248*, 588-595.

Teshima, Y. and Kakiuchi, S. (1974) Biochem.Biophys.Res.Commun. *56*, 489-495.

Tezuka, T. and Yamamoto, Y. (1972) Plant Physiol. *50*, 458-462.

Tezuka, T. and Yamamoto, Y. (1975) Plant Physiol. *56*, 728-730.

Thompson, E.W. and Lane, B.G. (1980) J.Biol.Chem. *255*, 5965-5970.

Timourian, H., Clothier, G. and Watchmaker, G. (1972) Exp.Cell Res. *75*, 296-298.

Timourian, H., Jotz, M.M. and Clothier, G.E. (1974) Exp.Cell Res. *83*, 380-386.

Toole, J.T., Hastie, N.D. and Held, W.A. (1979) Cell *17*, 441-448.

Torrey, J.G. (1953) Am.J.Bot. *40*, 525-533.

Torrey, J.G. (1955) Am.J.Bot. *42*, 183-198.

Torrey, J.G. (1957) Am.J.Bot. *44*, 859-870.

Torrey, J.G. (1965) Enc.Plant Physiol. *15*(I), 1256-1327.

Torrey, J.G. and Zobel, R. (1977) In *The Physiology of the Garden Pea*, eds. J.F. Sutcliffe and J.S. Pate), Academic Press, London, pp.119-152.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc.Nat.Acad. Sci. *76*, 4350-4354.

Trewavas, A.J. (1976) In *Molecular Aspects of Gene Expression in Plants*, (ed. J.A. Bryant) Academic Press, London, pp. 249-298.

Trewavas, A.J. (1979) In *Differentiation and the Control of Development in Plants*, (ed. E.C. George) BPGRG, Monograph No.3, Wessex Press, Wantage, pp.39-55.

Trewavas, A.J. (1982) In *The Molecular Biology of Plant Development* (eds. H. Smith and D. Grierson), Blackwell, Oxford, pp.7-27.

Tse, T.P.H. and Taylor, J.M. (1977) J.Biol.Chem. *252*, 1272-1278.

Tsien, R.Y. (1980) Biochem. *19*, 2396-2404.

Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature *295*, 68-71.

Ui, N. (1971) Biochim.Biophys.Acta *229*, 567-581.

Ui, N. (1973) Annals New York Acad.Sci. *209*, 198-209.

Vanaman, T.C. (1980) In *Calcium and Cell Function*, (ed. W.Y. Cheung) Vol.I, Academic Press, New York, pp.41-58.

Vanaman, T.C., Sharief, F., Awramik, J.L., Mendel, P.A., and Watterson, D.M. (1976) In *Contractile Systems in Non-muscle Cells*, (eds. S.V. Perry, A. Margreth, and S.Adelstein), Elsevier/North Holland Biomedical Press, New York, pp.165-176.

Van Belle, H. (1981) Cell calcium *2*, 483-494.

Van Brunt, J. and Harold, F.M. (1980) J.Bacteriol. *141*, 735-744.

Van Eldik, L.J., Grossman, A.R., Iverson, D.B. and Watterson, D.M. (1980a) Proc.Nat.Acad.Sci.U.S.A. *77*, 1912-1916.

Van Eldik, L.J., Piperno, G. and Watterson, D.M. (1980b) Proc. Nat.Acad.Sci.U.S.A. *77*, 4779-4783.

Van Eldik, L.J., Piperno, G. and Watterson, D.M. (1980c) Ann.New York Acad.Sci. *356*, 36-42.

Van Eldik, L.J. and Watterson, D.M. (1979a) J.Biol.Chem. *254*, 10250-10255.

Van Eldik, L.J. and Watterson, D.M. (1979b) Fed.Proc. *38*, 789.

Van Eldik, L.J. and Watterson, (1980) In *Calcium Binding Proteins: Structure and Function*, (eds. F.L. Siegel *et al.*) Elsevier, North Holland, pp. 247-250.

Van Fleet, D.S. (1952) Bot.Rev. *18*, 354-398.

Verma, D.P.S., Maclachlan, G.A., Byrne, H. and Ewings, D. (1975) J.Biol.Chem. *250*, 1019-1026.

Vesterberg, O. (1976) In *Isoelectric Focusing* (ed. N. Catsimpoolas) Academic Press, New York, pp.53-76.

Walden, R. and Leaver, C.J. (1978) In *Chloroplast Development*, (eds. G. Akoyunoglou and J.H. Argyroudi-Akoyunoglou, Elsevier, North Holland.

Walker, D.A. (1976) In *The Intact Chloroplast* (ed. J. Barber), Elsevier, North Holland, pp.235-278.

Wallace, R.W. and Cheung, W.Y. (1979) J.Biol.Chem. *254*, 6564-6571.

Wallace, R.W., Tallant, E.A. and Cheung, W.Y. (1980) Biochem. *19*, 1831-1837.

Walsh, M., Stevens, F.C., Oikawa, K. and Kay, C.M. (1979) Can.J.Biochem. *57*, 267-278.

Wang, A.H.-J., Quigley, G.J., Kolpak, F.J., Crawford, J.L., van Boom, J.H., van der Marel, G. and Rich, A. (1979) Nature *282*, 680-686.

Wang, J.H. and Desai, R. (1977) J.Biol.Chem. *252*, 4175-4184.

Wangermann, E. (1974) New Phytol. *73*, 623-635.

Wardlaw, C.W. (1965) Enc.Plant Physiol. *15*(1), 966-1076.

Wareing, P.F. (1979) In *Differentiation and the Control of Development in Plants-Potential for Chemical Modification* (ed. E.C. George), BPGRG Monograph No.3, Wessex Press, Wantage, pp.1-9.

Wareing, P.F. (1982) In *The Molecular Biology of Plant Development* (eds. H. Smith and D. Grierson), Blackwell, Oxford, pp.517-541.

Warren Wilson, J. (1978) Proc.Roy.Soc.Lond.Series B. *203*, 153-176.

Wasserman, W.J. and Smith, L.D. (1981) J.Cell Biol. *89*, 389-394.

Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J.Biol.Chem. *251*, 4501-4513.

Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980) J.Biol.Chem. *255*, 962-975.

Weber, C., Franke, W.W., Kartenbeck, J. (1974) Exp.Cell Res. *87*, 79-106.

Weber, C. and Kleinig, H. (1971) Planta *99*, 179-182.

Weber, K. and Osborn, M. (1969) J.Biol.Chem. *244*, 4406-4412.

Webster, P.L. and MacLeod, R.D. (1980) Env.Exp.Bot. *20*, 335-358.

Weir, E.M., Riezman, H., Grienenberger, J.-M., Becker, W.M. and Leaver, C.J. (1980) Eur.J.Biochem. *112*, 469-477.

Weiss, B., Fertel, R., Figlin, R. and Uzunov, P. (1974) Mol.Pharmacol. *10*, 615-625.

Weiss, B. and Levin, R.M. (1978) Adv.Cyclic Nuc.Res. *9*, 285-303.

Weisenseel, M.H., Dorn, A. and Jaffe, L.F. (1979) Plant Physiol. *64*, 512-518.

Weiss, B., Prozialeck, W., Cimino, M., Barnette, M.S. and Wallace, T.L. (1980) Ann.New York Acad.Sci. *356*, 319-345.

Wells, G.N. and Beevers, L. (1975) Arch.Biochem.Biophys. *170*, 384-391.

Welsh, M.J., Aster, J.C., Ireland, M., Alcala, J. and Maisel, H. (1982) Sci. *216*, 642-644.

Welsh, M.J., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1978) Proc.Nat.Acad.Sci. U.S.A. *75*, 1867-1871.

Welsh, M.J., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1979) J.Cell Biol. *81*, 624-634.

Wetmore, R.H., DeMaggio, A.E. and Rier, J.P. (1964) Phytomorph. *14*, 203-217.

Wetmore, R.H. and Rier, J.P. (1963) Am.J.Bot. *50*, 418-430.

Whaley, W.G., Mollenhauer, H.H. and Leech, J.H. (1960) Am.J. Bot. *47*, 319-349.

Whatley, J.M. and Gunning, B.E.S. (1981) New Phytol. *89*, 129-138.

Wick, S.M. and Hepler, P.K. (1980) J.Cell Biol. *86*, 500-513.

Wigle, D.T. and Smith, A.E. (1973) Nature New Biol. *242*, 136-140.

Williams, L.M., Jordan, E.G. and Barlow, P.W. (1983) Protoplasma, *118*, 95-103.

Williamson, A.R., Salaman, M.R. and Kreth, H.W. (1973) Ann. New York Acad.Sci. *209*, 210-224.

Wodzicki, T.J. and Brown, C.L. (1973) Am.J.Bot. *60*, 631-640.

Wolpert, L. (1979) In *Differentiation and the Control of Development in Plants-Potential for Chemical Modification* (ed. E.C. George), BPGRG Monograph No.3, Wessex Press, Wantage, pp.21-28.

Wong, W.-J.L. and Ap Rees, T. (1971) Biochim.Biophys.Acta *252*,296-304.

Wood, J.G., Wallace, R.W., Whitaker, J.N. and Cheung, W.Y. (1980) J.Cell Biol. *84*, 66-76.

Wooding, F.B.P. and Northcote, D.H. (1964) J.Cell Biol. *23*, 327-337.

Woodstock, L.W. and Skoog, F. (1962) Am.J.Bot. *49*, 623-633.

Wrigley, C.W. (1976) In *Isoelectric Focusing* (ed. N.Catsimpoolas), Academic Press, New York, pp.93-117.

Yalow, R.S. (1978) Sci. *200*, 1236-1245.

Yamada, M., Usami, Q. and Nakajima, K. (1974) Plant Cell Physiol. *15*, 49-58.

Yamada, M. and Usami, Q. (1975) Plant Cell Physiol. *16*, 879-884.

Yamamoto, Y. (1963) Plant Physiol. *38*, 45-54.

Yamanaka, M.K. and Kelly, L.E. (1981) Biochim.Biophys.Acta *674*, 277-286.

Yeoman, M.M. and Brown, R. (1971) Ann.Bot. *35*, 1101-1112.